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Impact of dietary calcium on vitamin D dietary requirements and bio-fortified food solutions for vitamin D deficiency

Thesis presented by:

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I wish to thank the funding bodies that made this research possible; the Department of Health, England and the Irish Department of Agriculture, Food and Marine under its Food Institutional Research Measures. Most importantly, I would like to extend my sincere thanks to the participants who took part in our studies for their interest and diligence, without them, this thesis would not have been possible.

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To my brother, Darragh, for always believing in me and for showing me that my opinion matters. I have been blessed to have a family who have impressed upon me the importance of listening and the knowledge to be gained from others. His ability to exert calmness upon every situation is something I admire. I want to thank him for always putting me on the right track. To Karen, for always giving me an opportunity to talk about my PhD, whether what I had to say was good or bad!

To Susannah and Tom, my niece and nephew, for never failing to make me laugh; I am so lucky to be around to see you growing up; you make me appreciate once again the wonder of learning. To see how you approach the world has given me a new perspective. How you can have so much joy from each day reminds me that the little things are the big things in life.

Working within the nutrition group at UCC has been one of the most fulfilling parts of my career so far. It has been a privilege to work with some amazing people with such a vast range of skills and all with the same goal - to produce the best science possible. I want to thank everyone here, whether it was a chat on the corridor or a cup of coffee, all of it makes this place, a wonderful place to work. I love that whenever I didn't know where to look next, Bernice always pointed me in the right direction. Thank you to both Bernice and Karen for always having an open door and being so genorous with their time. An extra special thank you to Karen, for explaining and re-explaining all things HPLC and to Alice for enlightening me about vitamin K. To those I have roomed with in 4.08, Kelly, Sinéad and Colette, thank you all for your advice and words of wisdom. I want to thank Kelly for believing in me form the beginning and reassuring me that this was the path for me; Sinéad, for showing me things that I only realise the value of now and, Colette for helping me see the end was in sight!

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Statement of Contribution

This thesis presents research from 2 separate randomised controlled trials (RCTs), 4 animal feeding trials, and data based on new analysis of biobanked serum from the most recent nationally representative nutrition survey, the National Adult Nutrition Survey (NANS), in Ireland. As such, the research was part of programme at the *Cork Centre for Vitamin D and Nutrition Research* at University College Cork (UCC) in collaboration with other groups. This research was funded in part by the UK, Department of Health and the Irish Department of Agriculture, Food and the Marine.

The following provides a concise overview of my contribution to the various research presented in this thesis:

Chapter 2 - Scientific searching, compilation of relevant literature, drafting and preparation of concise literature review, which forms basis of an invited review for *Proceedings of Nutrition Society* on foot of a selected overview presentation at the *Nutrition Society (NS) Irish Section Postgraduate Conference* in Cork in 2016.

Chapter 3 - Part A: conducted the *post-hoc* statistical analysis on data from our previous vitamin D RCTs and also on the update of our previous meta-analysis, under the supervision of my supervisor, Prof. Kevin Cashman. Data and statistical analysis, and presentation of data for inclusion in Final Project Report for the UK Department of Health.

Part B: Key member of a small research team that conducted the 'Vitamin D × Calcium' RCT in October, 2012- March 2013; with role in subject recruitment, food frequency questionnaire visits, all clinical visits, blood sample processing and bio-banked, logistics and shipment of bloods to Cork University Hospital for measurement of serum calcium and albumin, and I performed the analysis of serum 25(OH)D, 24,25 dihydroxyvitamin D [$24,25(OH)_2D$], 1,25 dihydroxyvitamin D, vitamin D binding protein, and parathyroid hormone via appropriate tandem liquid chromatography (LC-MS/MS) and immunoassay methodology; statistical analysis of data, and preparation of sections of the main resulting scientific paper, and also associated papers. Organisation of a participant evening event at which study participants got their dietary and vitamin D status data as well as a presentation by Kevin Cashman on vitamin D and health.

Part C: Identification of the relevant subset of NANS participants to fit with objectives of the analysis, retrieval of samples from biobank, and measurement

of serum 24,25$(OH)_2$D by LC-MS/MS. Data and statistical analysis, and presentation of data for inclusion in Final Project Report for the UK Department of Health.

Chapter 4 -There were several animal-feeding trials that investigated the potential of bio-fortification with vitamin D in laying hens, pigs and beef heifers, all part of a nationally funded collaborative project (Enhance*D* meats) between UCC (Coordinators) and University College Dublin. I was involved at the planning stage, some aspects of logistics, liaison with research staff from Lyons Research Farm in relation to the secure and timely transfer of eggs to UCC for analysis. Attended the industrial pig and beef cattle processing plants on the slaughter days to take blood samples and collect the correctly coded samples of pork and beef for ultimate vitamin D analytical analysis as well as the sensory trials in UCC. I undertook the analysis of porcine and bovine serum 25(OH)D and the egg, beef and pork vitamin D and 25-hydroxyvitamin D analysis by HPLC, following representative sampling, processing and extraction procedures. I also organised shipment of samples of all three foods to the Danish Technical University who undertook pooled sample analysis to inform decision-making on treatments to be included in animal feeding trials for production of food for the RCT in Chapter 5. Food sample storage prior to testing in sensory panels. Performed data and statistical analysis; drafting of Chapter 4 which is currently being converted into potentially two scientific papers in collaboration with colleagues in University College Dublin.

Chapter 5 - Led a small research team that conducted the RCT on the effectiveness of vitamin D bio-fortified eggs in December 2014 – March, 2015; with role in subject recruitment, food frequency questionnaire visits, all clinical visits, blood sample processing and bio-banking, logistics around weekly receipt of biofortified eggs from Lyons Research Farm at University College Dublin, their storage, and subsequent packaging and distribution of eggs to study participants; and performed the analysis of serum 25(OH)D, 24,25$(OH)_2$D, parathyroid hormone, serum calcium albumin and total cholesterol, via appropriate tandem liquid chromatography (LC-MS/MS), immunoassay and Clinical Chemistry Analyser methodology; I also performed the RCT egg confirmatory vitamin D and 25-hydroxyvitamin D analysis by HPLC; statistical analysis of data, and preparation of draft main scientific paper. Organisation of a participant evening event at which study participants got their dietary and

vitamin D status data as well as a presentation by Kevin Cashman on vitamin D and health.

Chapter 6 – Data on vitamin K intake in NANS were generated by the *Dietary Surveys Research* team at UCC (under direction of Professor Albert Flynn). I configured the newly acquired automated ELISA platform within the Centre for analysis of serum carboxylated and undercarboxylated osteocalcin in the biobanked NANS samples (*n* 692). I conducted the statistical analysis of the status results, but also intake and associational results under the guidance of my supervisor, Prof. Kevin Cashman and Dr. Alice Lucey. Preparation of draft Chapter 6, which has been submitted for peer review for scientific publication.

Declaration

This thesis is my own work and has not been submitted for another degree either at the National University of Ireland, University College Cork or elsewhere.

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Aoife Hayes

30th June 2016

Abstract

Recent re-evaluations of dietary reference intakes/values (DRI/DRV) for vitamin D on either side of the Atlantic have established intake requirements at the 97.5th percentile between 10 and 20 µg/d, and between 7.5 and 10 µg/d at the 50th percentile (overviewed in *Chapter 2*). National nutrition surveys indicate that mean habitual intakes of vitamin D in young and adult populations are typically in the range 3-7 µg/d. As vitamin D supplementation will not be effective at a population level because the uptake is generally low, creative food-based solutions are needed to bridge the gap between current intakes and these new requirement values, and so as to counteract vitamin D inadequacy in Europe and elsewhere.

While DRV/DRI for vitamin D are established on the premise that the needs of all other nutrients are being met, in practice this may not always be the case where calcium intakes, for example, can be inadequate for a significant portion of the population. The US Dietary Guidelines Committee has again recently identified both vitamin D and calcium as nutrients of public health concern. This is of consequence as there are some animal studies and older human data to suggest that a low calcium intake may lead to the increased degradation of serum 25-hydroxyvitamin D [25(OH)D], thus potentially increasing the dietary requirement for vitamin D. The US-based Institute of Medicine in their DRI report on calcium and vitamin D highlighted that the relationship between habitual calcium intakes and vitamin D activation and catabolism was a key knowledge gap. We performed *post-hoc* analysis of data from our previous placebo-controlled, dose-related, vitamin D randomized controlled trials (RCTs) and, in addition, undertook a new specifically designed double-blind, randomised, placebo controlled intervention trial (*Chapter 3*), to address this important area. Collectively, the data showed that dietary calcium does not interact with vitamin D_3 in terms of determining the response and catabolism of serum 25(OH)D during winter in older adults, and thus the dietary vitamin D requirement is not altered by typical calcium intakes within the population ranging from low to high. This data contributes to a sound scientific basis for development of not only DRV but also nutrition policy and supplementation/fortification programmes for ensuring adequate vitamin D status in the population.

Biofortification of food with vitamin D is a novel approach aimed at improving the dietary availability of vitamin D from the current food supply and by that means, increasing the distribution of vitamin D intakes in the population. This method, whereby the vitamin D content of the animal diet is increased and thus is incorporated into animal tissues, and ultimately consumed by humans, was tested in a number of animal feeding trials (in hens, pigs and beef heifers) which adhered to the EU upper allowable level of addition of vitamin D compounds to feedstuffs (*Chapter 4*). Vitamin D food analytics confirmed that the total

vitamin D activity of pork and beef meat, but particularly eggs, can be increased by this approach, and also the resulting food produce which had a good consumer acceptability profile, based on the data from sensory trials. For example, vitamin D biofortifed eggs were shown to have ~5 μg vitamin D per medium egg, which would supply half the IOM's estimated average requirement for vitamin D. Importantly, we also provided the first RCT evidence, to our knowledge, that consumption of seven of these vitamin D biofortified eggs per week, which does not conflict with healthy eating guidelines, helped to maintain serum 25(OH)D concentrations over winter months, and prevented the occurrence of vitamin D deficiency, as evidenced in a control group who consumed up to 2 commercial eggs per week (*Chapter 5*).

As new roles for vitamin K emerge beyond coagulation, there is increased interest in measuring vitamin K status in population-based studies, particularly, nationally representative samples. In this regard, the present work (*Chapter 6*) estimated phylloquinone intakes and, in addition for the first time assessed a biomarker of vitamin K status (percent undercarboxylated osteocalcin in serum [%ucOC]), in a representative sample of Irish adults aged 18-90 years. The data suggest that a significant portion of Irish adults may have intakes of vitamin K that are likely inadequate. This finding may have implications for bone health, as the present study showed an association between a serum %ucOC and a serum-based biochemical marker of bone resorption. Thus, strategies to increase phylloquinone intakes in the Irish population may need to be considered.

Overall, the collective findings of this thesis will contribute to advancement of knowledge in these particular fields of nutrition research.

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Chapter 1

Introduction, orientation and workflow of the thesis

The classic role of vitamin D is to regulate calcium absorption and homeostasis and thus, prevent the development of rickets in infancy, facilitate bone growth in children and, to help reduce risk of osteomalcia in adults (Jones *et al*, 1998). In more recent times, and stemming from the greater recognition of vitamin D's non-calcemic roles, low vitamin D status has been associated with cardiovascular outcomes, all-cause mortality, several types of cancer, amongst other health outcomes (Newberry *et al*, 2014). While there are many controversial issues in relation to the impact of vitamin D intake and status on human health, there is general agreement that prevention of vitamin D deficiency is a public health priority. However, vitamin D deficiency is evident worldwide (van Schoor & Lips, 2013), and recent data show that 13% of European individuals have serum 25-hydroxyvitamin D [25(OH)D] concentration <30 nmol/L on average in the year (Cashman *et al*, 2016). These first pan-European data to quantify the scale of vitamin D deficiency, using one of the most conservative international thresholds to define deficiency, again dramatically underscores the urgent need for responsive public health strategies to address this public health problem.

Vitamin D dietary requirement recommendations [known as *Dietary Reference Intakes* (DRI) in the US and *Dietary Reference Values* (DRV) in Europe] are crucial from a public health perspective in providing a framework for prevention of vitamin D deficiency and optimizing vitamin D status of individuals. Their development is an iterative process and is based on the knowledge that is available at the time (Cashman & Kiely, 2011). The explosion in scientific research in vitamin D and health over the last two decades has led to a dramatically increased evidence-base in relation to vitamin D and has prompted several regulatory agencies to re-evaluate their DRV/DRI (Cashman, 2015; and overviewed in *Chapter 2* of this thesis). At the conclusion of DRI and DRV development processes, the associated reports highlight key knowledge gaps that need to be addressed by research to inform future revisions of these vitamin D recommendations. The Institute of Medicine (IOM) in the US was the first in the modern era to complete their most comprehensive re-evaluation of vitamin D requirements. In their 2011 DRI report, the committee highlighted a considerable number of key knowledge gaps and associated research needs (IOM, 2011). Many of these identified key knowledge gaps in relation to vitamin D will impact on the work of other agencies in Europe, who likewise were about to begin the process of re-evaluation of their own vitamin D DRVs. We had noted in particular that the vitamin D DRI established by the IOM in 2011, as per convention, was based on the assumption that the requirement for dietary calcium was being achieved (IOM, 2011), however a significant portion of adult populations in Europe and North America fail to meet respective dietary calcium requirements (Flynn *et al*, 2009; IOM, 2011). IOM highlighted the potential for

inadequate calcium intakes to cause changes in the efficient handling of, or physiological response to, vitamin D that might not otherwise be present. This could translate into higher vitamin D dietary requirements. This priority research question framed the initial aim of my PhD's research programme, outlined in the *Workflow* diagram at the end of this section, namely to elucidate the relationship between habitual calcium intakes and the dietary vitamin D requirement. The research conducted to address this knowledge gap is presented in *Chapter 3* and contributed to several published papers.

As will be discussed in *Chapter 2*, the DRV/DRI established by several key agencies over the last 5 years, are important public health instruments and can act as vitamin D intake targets. The low dietary vitamin D intakes in European populations, as outlined comprehensively by Vinas *et al.* (2011), highlight the fact that the current food supply (even including current food fortification and vitamin D supplementation practices) is not enough to meet these dietary vitamin D targets. These low intakes undoubtedly contribute to the wide-scale low vitamin D status in Europe (Cashman *et al*. 2016). Thus, strategies to increase the distribution of vitamin D intakes and reduce the prevalence of vitamin D deficiency in the population are paramount. Such approaches include: encouraging greater consumption of vitamin D-rich foods, vitamin D supplementation and food fortification policies, and potentially including 'biofortification'. Natural sources of vitamin D in the diet are limited and many are infrequently consumed (Black *et al*, 2014), thus recommendations to increase intakes of current vitamin D sources are unlikely to succeed in improving vitamin D intakes. Supplementation has been shown to significantly improve serum 25(OH)D concentrations in a dose-related manner, across population sub groups (Cashman & Kiely 2011; Cashman *et al*. 2011; Whiting *et al*. 2015). However, supplementation with vitamin D, while effective in those who consume them, is not a suitable public health policy, as uptakes are typically lower than 40% in the population (Fulgoni *et al*. 2011; Whiting *et al*. 2011; Black *et al*. 2015). Current fortification practices do significantly contribute to vitamin D intakes but are not effective at increasing vitamin D intakes across the population. For example, Fulgoni *et al*. (2011) reported using NHANES (2003-2006) data that 69.5% of the US population over 2 years of age did not achieve the Estimated Average Requirement (EAR) of 10 μg/d set by IOM, even after accounting for the contribution of supplement usage and consumption of vitamin D fortified foods. The percentage of the population with intakes below the EAR is an indicator of the degree of inadequacy of nutrient intake in that population. Two systematic reviews of available RCT data have highlighted that food fortification is effective in increasing serum 25(OH)D concentrations (O'Donnell *et al*, 2008; Black *et al*, 2012), and this may be the best approach to tackling inadequate vitamin D intakes across the

population. However, it has also been suggested that the selection of available fortified foods should be expanded to accommodate diversity in the diet (Vantanparast *et al*, 2010; Black *et al*, 2012; Cashman & Kiely, 2016). In this regard, there is a need for creative food-based solutions that are effective across the whole population.

Biofortification of food with vitamin D is a novel approach aimed at improving the dietary availability of vitamin D from the current food supply and by that means, increasing the distribution of vitamin D intakes in the population. In this approach, animal produce (such as, for example, cultured fish, beef, pork, lamb, chicken, and eggs) could have increased vitamin D and/or 25-hydroxyvitamin D contents by virtue of addition of vitamin D and/or 25-hydroxyvitamin D (where permissible) to the livestock feeds. This method also has the benefit of improving the levels of 25-hydroxyvitamin D in the food, in addition to vitamin D *per se*, which could boost the overall effectiveness of these foods in raising vitamin D status (Cashman *et al*, 2015). Findings from numerous animal feeding trials (in hens, pigs and cattle) form a very strong evidence-base to illustrate that this biofortification approach leads to animal produce (eggs and meat) with much improved vitamin D content (as reviewed in *Chapter 2*), however, the difficulty with this evidence-base is that the majority of these previous studies in hens, pigs or cattle included levels of vitamin D and/or HyD® (as commercially available 25-hydroxyvitamin D) in the feed in excess of the EU allowable maximum levels for use in respective feeds. Accordingly, *Chapter 4* of the present thesis focuses on the feasibility of biofortification of animal sources with vitamin D, using levels of vitamin D_3 and/or 25-hydroxyvitamin D_3 (commercially available as HyD®) that comply with EU regulations, and whereby the resulting foods would be fit for human consumption. The impact of several combinations of vitamin D and/or HyD®, where permissible, on vitamin D content of the resulting produce was determined through a series of animal trials in laying hens, pigs and beef heifers performed in Lyons Research Farm in collaboration with our colleagues in University College Dublin. Provision of evidence of effectiveness of food fortification approaches from randomized controlled trails (RCTs) which evaluate their impact on reducing the prevalence of vitamin D deficiency in the populations studied is a key priority in terms of the evidence-base (Cashman & Kiely, 2011). Thus, *Chapter 5* in the present thesis presents our new RCT data (just accepted for publication) on the effectiveness of vitamin D biofortified eggs (arising from the work in *Chapter 4*) in protecting against low serum 25(OH)D concentrations in adults in winter.

In a separate and final piece of my PhD's research programme, we aimed to gain some insight into vitamin K status in a nationally representative sample of Irish adults (*Chapter 6*). This was in response to the greater recognition of the new roles for vitamin K beyond

coagulation (Booth, 2009), and accordingly the increased interest in measuring vitamin K status in population-based studies, particularly, nationally representative samples.

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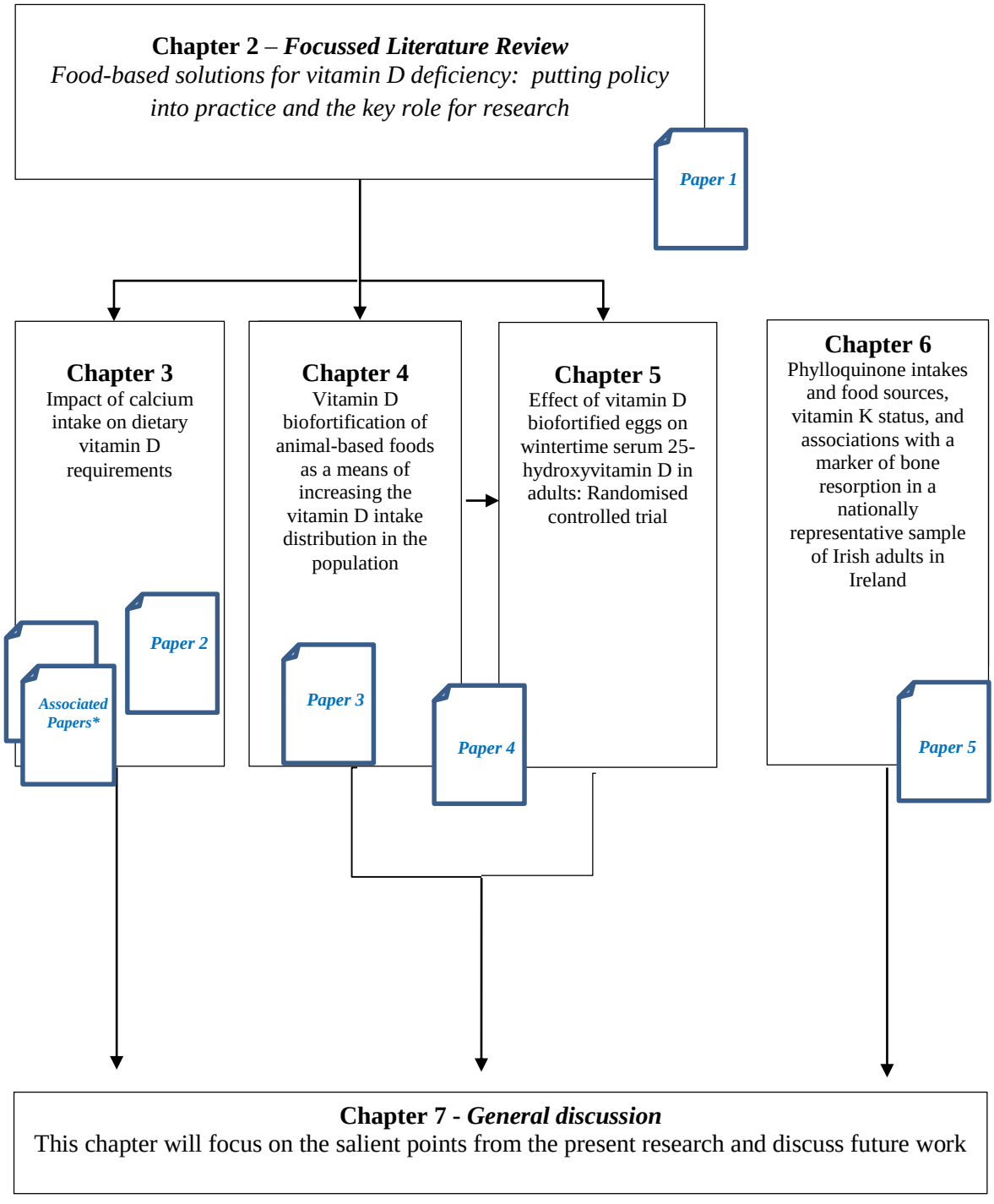
Workflow of thesis, including publication and presentation of its findings



Key to Papers, and also presentation of the data at national and international conferences

Paper 1: A. Hayes, K.D. Cashman. Food-based solutions for vitamin D deficiency: putting policy into practice and the key role for research. *Proc Nutr Soc* (in review).

[Invited review following Aoife Hayes: Winner of *Nutrition Society (Irish Section) Postgraduate Review* presentation 'Addressing key knowledge gaps in terms of developing food based solutions for prevention of vitamin D deficiency' in February, 2016; Cork]

Paper 2: K.D.Cashman, **A. Hayes**, S.M. O'Donovan, J.Y. Zhang, M. Kinsella, K. Galvin, M.Kiely, K.M. Seamans. Dietary Calcium does not interact with vitamin D3 in terms of determining the response and catabolism of serum 25-hydroxyvitamin D_3 during winter in older adults. *Am J Clin Nutr* 2014; 99:1414-23.

Associated peer-reviewed abstracts for conference presentations

A. Hayes, S.M. O'Donovan, J.Y. Zhang, M. Kinsella, K. Galvin, M.Kiely, K.M. Seamans, K.D. Cashman. Vitamin D Economy?—Dietary calcium does not interact with vitamin D_3 in terms of determining the response and catabolism of serum 25-hydroxyvitamin D during winter in older adults. *Nutrients* 2014; 6: Abstract 2.16. p. 2773.

[Aoife Hayes: Young Investigator award, *Vitamin D and Human Health: from the Gamete to the Grave* conference, April, 2014; London]

A. Hayes, K.M. Seamans, M. Kiely, K.D. Cashman. Effect of habitual calcium intake on dietary vitamin D requirements in adults. *Proc Nutr Soc* 2013; **72**: OCE3, E172.

***Associated papers** (in which additional vitamin D metabolite data from chapter 2 was used):* K.D. Cashman, **A Hayes**, K. Galvin, J Merkel, G. Jones, M. Kaufmann, A N. Hoofnagle, G D. Carter, RA. Durazo-Arvizu, C T. Sempos. Significance of serum 24,25-dihydroxyvitamin D in the assessment of vitamin D status: A double-edged sword? *Clin Chem* 2015;61:636-45.

K.D. Cashman, M. Kinsella, J. Walton, A. Flynn, **A. Hayes**, A. Lucey, K.M. Seamans, M. Kiely. The 3 Epimer of 25 hydroxycholecalciferol is present in the circulation of the majority of adults in a nationally representative sample and has endogenous origins. *J Nutr* 2014;144:1050-7.

Paper 3: Investigation of strategies for production of vitamin D biofortifed beef and pork (paper in preparation)

Paper 4: A. Hayes, S. Duffy, M. O'Grady, J. Jakobsen, K. Galvin, J. Teahan-Dillon, J. Kerry, A. Kelly, J. O'Doherty, S. Higgins, K.M. Seamans, K.D. Cashman. Vitamin D-enhanced eggs are protective of wintertime serum 25-hydroxyvitamin D in a randomized controlled trial of adults. *Am J Clin Nutr* 2016 (in press).

Associated peer-reviewed abstracts for conference presentations

A. Hayes, M. O'Grady, S. Duffy, K.M. Seamans, J. Kerry, A. Kelly, J. Jakobsen, J. O'Doherty, K.D. Cashman. The vitamin D content and consumer acceptability of vitamin D biofortified eggs. *Proc Nutr Soc* 2015; 74: OCE4, E243.

K. Cashman, S. Duffy, **A. Hayes**, K. Seamans, J. Kerry, A. Kelly, J. Jakobsen, J. O'Doherty. Biofortification of eggs and pork with vitamin D as a means of increasing dietary supply. *FASEB Journal* 2015; 29: 758.13 (abstract)

Paper 5: A. Hayes, Á. Hennessy, J. Walton, B.A. McNulty, A.J. Lucey, M. Kiely, A. Flynn, K.D. Cashman Phylloquinone intakes and food sources, vitamin K status, and associations with a marker of bone resorption in a nationally representative sample of Irish adults. (submitted *J Nutr*)

Associated peer-reviewed abstracts for conference presentations

A. Hayes, Á. Hennessy, J. Walton, B. McNulty, M. Kiely, A.Flynn, K.D. Cashman, A.J Lucey. Associations between vitamin K status and skeletal and cardio-metabolic health indices in 18-64-year-old Irish adults. *Proc Nutr Soc* 2015; 74: OCE4, E229

[Aoife Hayes: Best overall poster, *Nutrition Society Summer Conference (Irish Section).* 'Associations between vitamin K status and skeletal and cardio-metabolic health indices in 18-64-year-old Irish adults.' June, 2015, UCC, Cork].

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Chapter 2

Food-based solutions for vitamin D deficiency: putting policy into practice and the key role for research

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Aoife Hayes & Kevin D. Cashman

Proceedings of the Nutrition Society (invited review)

[in peer review]

2.1 Introduction

There is widespread acknowledgement of the presence of vitamin D deficiency in the community and the pressing need from a public health perspective to address this deficiency (Cashman & Kiely, 2011). Taking a serum 25-hydroxyvitamin D [25(OH)D] concentration of 30 nmol/L as the cut-off below which the risk of clinical vitamin D deficiency increases (manifesting as nutritional rickets in children and osteomalacia in adults) (IOM, 2011; Munns *et al*, 2016), prevalences of vitamin D deficiency based on standardized serum 25(OH)D data from representative population samples in the United States (US) (*n*=15,652) (Schleicher *et al*, 2016), Canada (*n*=11,336) (Sarafin *et al*, 2015) and Europe (*n*= 55,844) (Cashman *et al*, 2016a) have been recently reported as 5.9%, 7.4% and 13%, respectively. Even a crude estimation based on the magnitude of populations in these three regions and the above prevalences suggests something in the region of 120 million individuals are deficient.

Dietary reference intervals for vitamin D, which are estimates of the dietary requirements for vitamin D, are crucial from a public health perspective in providing a framework for prevention of vitamin D deficiency and optimizing vitamin D status of individuals (Cashman, 2015). While in North America the term used to describe the distribution of dietary requirements is Dietary Reference Intakes (DRI), its equivalent in Europe is Dietary Reference Values (DRV) (Cashman, 2012a). An enormous body of research in relation to various aspects of vitamin D and health over the last decade and a half has been instrumental in informing recent DRV/DRI for vitamin D (Cashman, 2015). While there are a family of nutrient reference values within the DRI and DRV, two shared constituent values within both, and which are of key public health importance, are the Estimated Average Requirement (EAR) and the Recommended Dietary Allowance (RDA; or its equivalent). The EAR is the level of (nutrient) intake that is adequate for half of the people in a population group, and also serves as the basis for calculating the RDA (or equivalent), a value intended to meet the needs of nearly all (i.e., 97.5%) healthy individuals in a population (IOM, 2011; EFSA, 2010). Despite the fact that the DRI/DRV for vitamin D have been re-evaluated on either side of the Atlantic in the last 5 years (Cashman, 2015), and have resulted in quite contrasting recommendations (see below), nevertheless, they collectively point very strongly towards a need for creative food-based approaches for prevention of vitamin D deficiency and maintenance of nutritional adequacy. However, persistent knowledge gaps are barriers to developing and implementing safe and effective food-based approaches towards prevention of vitamin D deficiency (Cashman & Kiely, 2015). The present review will first of all concisely overview current DRI/DRV for vitamin D as key dietary targets. It will then also briefly benchmark current

intakes of vitamin D in selected representative samples in Europe and North America against these dietary targets, and, finally, it will consider means of addressing the gap between current intakes and these dietary targets. In particular, focus will be placed on the area of fortification of food with vitamin D, and especially 'bio-fortification' with vitamin D, as a means of increasing the distribution of vitamin D intake in the population to prevent vitamin D deficiency. These three aspects of dietary vitamin D in the present review were the central thrust of one of the authors' (AH) accompanying presentation at the *Nutrition Society (NS) Irish Section Postgraduate Conference* in Cork in 2016.

2.2 Dietary Reference Intakes/Values from North America, the United Kingdom and European Union: variable estimates of dietary vitamin D requirement

It is important to re-iterate that devising nutrient recommendations for population intakes relies on scientific analysis and judgement of data that exist within a specified time frame and is an iterative process (Cashman & Kiely, 2011). It is notable in that context, that within the last five years there have been re-evaluations of vitamin D recommendations by at least 6 expert bodies on either side of the Atlantic, and four of these have been reviewed in detail elsewhere (Cashman, 2012a; 2015; Cashman & Kiely; 2014; 2016). For this present work, we will focus on the two very most recent DRV in Europe, namely those from the Scientific Advisory Committee on Nutrition [SACN] in the United Kingdom (UK) (SACN, 2016) and the European Food Safety Authority [EFSA] (EFSA, 2016), as well as referencing the DRI from the Institute of Medicine [IOM] in the US (IOM, 2011), as their most current recommendations but also a landmark evaluation and report in the current era of dietary requirement estimates for vitamin D. Of note, despite being based on a largely similar underpinning scientific evidence-base, there is significant variation in the recommendations from these 3 sets of DRI/DRV (see below). It is not within the scope of the present review to explore in detail all the reasons why the recent DRI/DRV differ, but in the context of considering food-based solutions for prevention of vitamin D deficiency, it is worth summarizing briefly what these 3 agencies established as the DRI/DRV and also based on what criteria to establish nutrient adequacy for vitamin D. All 3 agencies used serum 25(OH)D as the biochemical indicator of vitamin D exposure/status (IOM, 2011; SACN, 2016; EFSA, 2016). This is not surprising as it has been shown to be a robust indicator of vitamin D status (Seamans & Cashman, 2009), albeit with some potential limitations (Cashman & Kiely, 2011). All three agencies adhered to the risk assessment framework for DRI/DRV development and, as such, following a review of the evidence-base in relation to vitamin D and health (skeletal and non-skeletal) effects, all three agencies used (musculo-)skeletal health outcomes (and in addition EFSA used pregnancy-related health outcomes)

upon which to base the development of their respective DRI/DRV (IOM, 2011; SACN, 2016; EFSA, 2016). The IOM's DRI committee selected calcium absorption, bone mineral density (BMD) and either rickets in children or osteomalacia in adults, for which the evidence was sufficiently strong, for DRI development for vitamin D (IOM, 2011). SACN selected musculoskeletal health (rickets, osteomalacia, falls, muscle strength and function, depending on age group) (SACN, 2016), whereas EFSA considered that the available evidence on serum 25(OH)D concentration and musculoskeletal health outcomes and pregnancy-related health outcomes were suitable to set DRVs for vitamin D for adults, infants, children, and pregnant women, respectively (EFSA, 2016).

Even though the DRI/DRV were majorly influenced by bone-related health outcomes, the choice of these different individual outcomes, either collectively or individually, influenced the selection of serum 25(OH)D concentration(s) upon which to base the DRI/DRV (**Table 1**). The IOM's DRI committee established a serum 25(OH)D concentration of 40 nmol/L as the median value above which approximately half the population might meet its vitamin D requirement in terms of bone health (and below which half might not; and which formed the basis for establishment of their EAR) and 50 nmol/L as its estimate of the serum 25(OH)D concentration that would meet the requirement of nearly all (i.e. 97.5 %) "normal healthy persons", and which thus formed the basis of the establishment of their RDA (IOM, 2011). The EFSA panel concluded that a serum 25(OH)D concentration of 50 nmol/L is a suitable target value to set the DRVs for vitamin D, for all age and sex groups (adults, infants, children, pregnant and lactating women) (EFSA, 2016). In contrast, SACN considered that the evidence overall suggested the risk of poor musculoskeletal health was increased at serum 25(OH)D concentrations below ~20-30 nmol/L (SACN, 2016). They felt it was not possible to identify a specific serum 25(OH)D threshold concentration between 20-30 nmol/L associated with increased risk of poor musculoskeletal health due to different analytical methods for serum 25(OH)D used in the various studies, so decided to retain the previously used 25 nmol/L (SACN, 2016). This previous threshold was used to indicate increased risk of vitamin D deficiency as relates to rickets and osteomalacia (DOH, 1991), which is largely consistent with the 30 nmol/L which the IOM DRI committee had indicated reflects increased risk of vitamin D deficiency (IOM, 2011). Furthermore, SACN suggested that the use of 25 nmol/L represents a 'population protective level' in that it is a concentration that individuals in the UK should be above, throughout the year, in terms of protecting musculoskeletal health (SACN, 2016).

Table 1. Selected Dietary Reference Values/Intakes for vitamin D*.

DRV/DRI….	*UK RNI*	*EFSA AI*	*North American*	
			EAR	*RDA*
Serum 25(OH)D target (nmol/L)	≥25	≥50	≥40	≥50
Age group		(μg/d)[†]		
0-6 months	8.5-10[¶]	-	-	10[‡]
7-12 months	8.5-10[¶]	10	-	10[‡]
1-3 years	10[¶]	15	10	15
4-6 years	10	15	10	15
7-8 years	10	15	10	15
9-10 years	10	15	10	15
11-14 years	10	15	10	15
15-17 years	10	15	10	15
18-64 years	10	15	10	15
65+ years	10[¶]	15	10	15 (20 for 70+ years)
Pregnancy	10[¶]	15	10	15
Lactation	10[¶]	15	10	15

DRI, Dietary Reference Intakes; DRV, Dietary Reference Values/; RNI, Reference Nutrient Intake; AI, Adequate Intake; RDA, Recommended Dietary Allowance; EAR, Estimated Average Requirement; 25(OH)D, 25-hydroxyvitamin D

[*]All established under conditions of minimal cutaneous vitamin D synthesis

[†]To convert vitamin D reference intakes from μg/d to IU/d, multiply by 40.

[¶]Set as a Safe Intake; set for some nutrients if there were insufficient data to set DRVs. They are judged to be a level or range of intake at which there is no risk of deficiency, and below a level of where there are undesirable effects (DoH, 1991).

[‡]Set as Adequate Intake.

Using these various serum 25(OH)D target concentrations, the 3 expert bodies were able to derive dietary requirement estimates which form their respective DRI/DRV for vitamin D Both IOM and EFSA used a meta-regression approach on data from winter-based vitamin D randomized controlled trials (RCTs) which reported serum 25(OH)D response (IOM, 2011; EFSA, 2016), while SACN used requirement estimates derived from analysis and modelling of individual subject-level data from what they considered as 3 highly UK-appropriate vitamin D dose-related RCTs (Cashman *et al*, 2008; 2009; 2011a), all of which focussed on the estimation of vitamin D dietary requirements in different population age-groups. The pros and cons of these two different approaches have been well versed elsewhere (Cashman *et al*, 2011b; IOM, 2011; Cashman, 2015; SACN, 2016; EFSA, 2016), but undoubtedly contribute to intake estimates which differ dramatically, even if one uses the same serum 25(OH)D target concentration (Cashman *et al*, 2011b). Irrespective of this, it is notable that while all three agencies have an EAR and RDA (or equivalent) available within their family of respective DRV/DRI, the IOM established EAR and RDA values for most age-groups (IOM, 2011), while SACN established only a Reference Nutrient Intake (RNI, an RDA equivalent) for most age-groups but not an EAR (because there is never as high as 50% with serum 25(OH)D <25 nmol/L, prohibiting its estimation) (SACN, 2016), and EFSA established Adequate Intake values in place of either EARs or Population Reference Intakes (PRI; their RDA equivalent) (EFSA, 2016) (Table 1). EFSA in their 2010 scientific opinion on principles for deriving and applying DRV had proposed that an AI should be derived when a PRI cannot be established for a nutrient because an EAR cannot be determined (EFSA, 2010). An AI is the average observed daily level of intake by a population group (or groups) of apparently healthy people that is assumed to be adequate (EFSA, 2010). To illustrate the variability in the current recommendations, if one selects adults aged 18-64 years as an example, the vitamin D intakes established to ensure the adequacy of the vast majority of individuals (97.5%) in either North America or the European Union are 15 µg/d (with serum 25(OH)D at 50 nmol/L as the target), 10 µg/d will meet the needs of half the individuals in North America (again at 50 nmol/L as the target), whereas in the UK, 10 µg/d will ensure the adequacy of the vast majority of individuals but using serum 25(OH)D at 25 nmol/L as the target (see Table 1).

Not only do the types of DRV/DRI that the 3 different agencies established differ, so too does their application from a public health nutrition perspective in terms of assessing the risk of (in)adequacy of nutrient intake in populations. For example, EFSA in their 2010 opinion on general principles for development and application of DRV suggest that the EAR can be used to estimate the prevalence of inadequate intakes of micronutrients (the Average Requirement cut-point method), if the distribution of nutrient intakes is normal, and intakes are independent

from requirements (EFSA, 2010). The percentage of the population with a habitual daily nutrient intake that is lower than the EAR is taken as an estimate of the percentage of the population with probable inadequate intakes. Thus, the IOM established EAR (IOM, 2011) can be utilized in this context in terms of nutritional surveillance. The RDA (and RNI/PRI) is an intake level that covers the requirement of 97.5% of all individuals when requirements of the group have a normal distribution (IOM, 2011; SACN, 2016; EFSA, 2016), it should therefore not be used as a cut-point for assessing nutrient intakes of groups because a certain overestimation of the proportion of the group at risk of inadequacy would result (EFSA, 2010). The current North American RDA (IOM, 2011) and UK RNI (SACN, 2016), however, will have application in terms of assessing diets of individuals (EFSA, 2010; Cashman, 2012a). Groups with mean intakes at or above the AI (as established by EFSA and for infants and/or young children by IOM and SACN) can be assumed to have a low prevalence of inadequate intakes for the defined criterion of nutritional status (EFSA, 2010). Higher intakes convey no additional health benefit and in fact if they exceed the tolerable upper level may lead to harm (EFSA, 2010).

2.3 A clear public health lesson from these disparate DRV/DRI

Notwithstanding the variability in current DRV/DRI, benchmarking European population intakes against a vitamin D intake value of 10 μg/d, corresponding both to the North American EAR (IOM, 2011) and the RNI in the UK (SACN, 2016), shows that there are between 55-100% of adults (19-64 y) and older adults (>64 y) with inadequate intakes (Vinas *et al*, 2011) on this basis. Typical average intakes by adult populations in the EU are generally around 3-7.5 μg/d, depending on the country (Vinas *et al*, 2011). The European Nutrition and Health Report summarized vitamin D intake in children and teenagers (aged 4-14 y) and showed that intakes were in the range 1.2-6.5 μg/d (Elmadfa *et al*, 2009). The dietary intakes of children and adults in North America and some European countries have been comprehensively reviewed elsewhere by Kiely & Black (2012), and the readers are referred to that review for more detailed analysis of population intakes of vitamin D from select countries relative to the EAR. The estimates clearly show that the habitual mean vitamin D intakes by European and North American populations are below the estimates of intake requirement.

While at first glance recommending improving intake of naturally occurring vitamin D-rich foods would appear intuitive, it is the least likely strategy to counteract low dietary vitamin D intake due to the fact that there are very few food sources that are rich in vitamin D (Kiely & Black, 2012; Cashman 2015). Furthermore, most of these are not frequently consumed by many in the population (Henderson *et al*, 2004; Kiely & Black, 2012; Black *et al*, 2015;

SACN, 2016). While there have been calls for use of vitamin D supplements as a means of correcting low vitamin D status in European populations, the principal reason why relying on supplements is not an appropriate public health strategy to increase vitamin D intakes across the population distribution, i.e., uptake within the population being generally too low to provide widespread population protection, has been outlined elsewhere (Cashman, 2012b; Cashman & Kiely, 2015; Kiely & Cashman, 2016). Data from three nationally representative cross-sectional dietary surveys in Ireland (a survey in children (*n*=594; 5–12 years) (Black *et al*, 2014), teenagers (*n*=441; 13–17 years) (Black *et al*, 2014) and adults (*n*=1274; 18-64 y) (Black *et al*, 2015), illustrate this point very clearly. The surveys showed that 97–99% of all boys, 98–99% of all girls, and 90% of all adults had inadequate intakes of vitamin D, as defined as ≤10 μg/d. The percentage of adults who consumed vitamin D–containing supplements was only 16% (Black *et al*, 2015*)*, and a total of 21% of 5–8-year-olds, 16% of 9–12-year-olds and 15% of 13–17-year-olds consumed a vitamin D-containing supplement at least once during the recording days of their survey (Black *et al*, 2014). Moreover, even in users of vitamin D-containing supplements within the three surveys, 88–96% of those boys and 88–90% of those girls, and 57% of those adults (aged 18-64 y) with supplemental vitamin D had inadequate intakes (Black *et al*, 2014; 2015).

In comparison to vitamin D supplementation or relying on improving intake of naturally occurring vitamin D-rich foods, food fortification may represent the best opportunity to increase the vitamin D supply to the population, even though it is not also without its challenges. Fortification of foods with vitamin D in the US and Canada has an important effect on the mean daily intake of vitamin D by the average adult; however, Calvo & Whiting (2013) suggest that the current level of fortification in the US and Canada is not effective in reaching the required levels of vitamin D intake. Fulgoni *et al*. (2011) showed using data from NHANES 2003-2006 that the percentage of individuals in the US aged 2 and older below the EAR was reduced when the contribution of vitamin D-fortified foods was accounted for, but still 69.5% did not reach the EAR. The adult nutrition survey in Ireland, mentioned above, recently showed that the percentage failing to meet the EAR was 98% in non-consumers of vitamin D supplements or vitamin D-fortified foods, and 95% in non-consumers of vitamin D supplements but who consumed vitamin D-fortified foods (Black *et al*, 2015). Flynn *et al*. (2009) have shown that the 95th percentile of intake of vitamin D from voluntary fortified foods in Europe is low (Flynn *et al*, 2009). These findings showing the relatively low impact of vitamin D-fortified foods to date may relate to the level of fortification, types and choice of food vehicles and the issue of mandatory or optional/voluntary fortification (Kiely & Black, 2012; Cashman, 2015) as well as market penetrance of voluntary fortified foods. The remainder of the review will focus on how careful application of traditional food fortification

(nutrient addition in controlled amounts) and 'bio-fortification' (enrichment of animal food sources, such as meats, eggs, and fish, as well as of mushrooms) strategies could be used to safely increase intakes of vitamin D across the distribution and prevent deficiency within the general population. In addition, it will highlight the ongoing need and role research has in terms of addressing persistent knowledge gaps that exist and which are barriers to developing and implementing such safe and effective food-based approaches towards prevention of vitamin D deficiency.

2.4 Using food-based solutions for increasing vitamin D intake in the population

While under legislation in North America and Europe, several food types can be fortified, milk and margarine are the foods with the longest tradition of fortification (Cashman & Kiely, 2016). Canada currently has mandatory fortification of milk and margarine with vitamin D as stipulated by the Canadian Food and Drug Regulations, and while fluid milk in the US is not required to have vitamin D added unless the label declares that it is fortified, in practice almost all milk is fortified with vitamin D on a voluntary fortification basis (Calvo & Whiting, 2013). Currently within Europe, fortification practices vary between countries and may be applied voluntarily by manufacturers or implemented by national legislation. The evidence-base for effectiveness of vitamin D fortification of milk and other dairy food in terms of its impact on vitamin D intake and serum 25(OH)D has been reviewed elsewhere (Black *et al*, 2012; Cashman & Kiely, 2016), and ranges from supportive data from several RCTs to evidence from modeling of population intakes and status. Overall, there is little doubt that vitamin D-fortified dairy foods play a key role in addressing low vitamin D intakes, especially where there is mandatory fortification (Cashman & Kiely, 2016). However, even in Canada with mandatory fortification, recent modeling of their nationally representative data (2004 Canadian Community Health Survey 2.2 with n=34,381) showed that the prevalence of dietary inadequacy of vitamin D could be decreased from >80% to <50% in all groups with increased vitamin D levels in milk and the addition of vitamin D to cheese and yogurt at various levels (Shakur *et al*, 2014). Furthermore, in countries where fortification of milk is voluntary and the uptake is far less than in the US, the impact of vitamin D-fortified milk and dairy on adequacy of intake of the vitamin is understandably low. The problem of fortifying a single staple, e.g., milk, or focusing on a commodity sector such as dairy, is that it does not increase the vitamin D supply in non- or low consumers (Black *et al*, 2012). Thus, while acknowledging the valuable contribution fortified milk makes to vitamin D intakes among consumers, particularly in children, and the continued need for fortification of milk and other dairy products, additional strategic approaches to fortification, including bio-fortification, of a wider range of

foods, have the potential to increase vitamin D intakes in the population (Cashman & Kiely, 2016).

2.5 Traditional fortification of foods other than dairy with vitamin D

In terms of diversification of food fortification beyond milk, Madsen *et al*. (2013) recently provided experimental evidence, in the form of RCT data, of the effects of vitamin D-fortified milk and bread on serum 25(OH)D in 201 families (*n*=782 children and adults, aged 4–60 years) in Denmark during winter. Bread was included as an additional vehicle for fortification in recognition of the skewness of milk intake across some population groups. The groups randomized to vitamin D unfortified and fortified foods had median intakes of vitamin D of 2.2 and 9.6 μg/d, respectively over the 6 months of the study. By the end of the study period, none and 16% in the fortified food group had serum 25(OH)D levels below 25 and 50 nmol/L, respectively, with the corresponding prevalence estimates for the group receiving unfortified foods at 12 and 65%, respectively (Madsen *et al*, 2013). Evidence of effectiveness of food fortification approaches from RCTs which evaluate their impact on reducing the prevalence of vitamin D deficiency in the populations studied is a key priority (Cashman & Kiely, 2011), but also dietary modelling analysis based on data from nationally representative dietary surveys can provide *in silico* projections of how these food interventions may impact on the degree of vitamin D intake inadequacy in the population (Black *et al*, 2015). For example, Allen *et al.* (2015) recently modeled the impact of a number of simulated vitamin D fortification scenarios, with milk and wheat flour identified as primary fortification vehicles, on vitamin D intake distribution within the first 2 years (2008-2010) of the UK National Diet and Nutrition Survey rolling programme (*n*=2127 individuals). At a simulated fortification of 10 μg vitamin D/100 g wheat flour, the proportion of at-risk groups estimated to have vitamin D intakes below the UK RNI was reduced from 93% to 50% (Allen *et al*, 2015). Interestingly, the simulation of the fortification of wheat flour at this concentration was more effective than that of the fortification of milk (at concentrations between 0.25 and 7 mg vitamin D/100 L milk) or of the fortification of milk and flour combined. The authors suggested that vitamin D fortification of wheat flour could be a viable option for safely improving vitamin D intakes and the status of the UK population groups at risk of deficiency (Allen *et al*. 2015)

The mandatory fortification of a food vehicle other than milk or margarine raises an interesting issue. A general consideration underpinning the establishment of DRV/DRI is that there is the presupposition that in setting the requirements for one nutrient, requirements for energy and all other nutrients are met (IOM, 2011; SACN, 2016; EFSA, 2016). For example, despite the

strong interactions between vitamin D and calcium, the DRV/DRI for vitamin D are set on the assumption that requirements for calcium are met and *vice versa*. In reality, however, this does not happen, since a significant proportion of populations in the US and Europe have dietary calcium intakes that are inadequate (Flynn *et al*, 2009; IOM, 2011). In fact, the US *2015-2020 Dietary Guidelines for Americans* Committee identified both vitamin D and calcium as two of the four identified nutrients of public health concern (DHSS, 2015). The IOM in their DRI report on calcium and vitamin D highlighted that there was ambiguity in the available data on the influence of dietary calcium intakes on regulation of serum 25(OH)D and its catabolism (IOM, 2011). This is of consequence when considering vitamin D fortification policies, due to the inter-relationship that exists between calcium and vitamin D in the body, and raises the question of whether the range of foods for vitamin D fortification should have calcium naturally present (e.g. dairy produce) or if both of these nutrients should be added in tandem.

There have been animal data that suggested that the plasma half-life of 25(OH)D will be reduced by low calcium intake accompanied by an increased hepatic conversion of 25(OH)D to metabolites, which are excreted in bile (Fraser *et al*, 1987) , thus potentially increasing the dietary vitamin D requirement. Indeed, calcium intake has been reported as a significant determinant of serum 25(OH)D in some, but not all observational studies (Lips *et al*, 2012). One RCT in healthy men reported a large increase (30%) in serum 25(OH)D in subjects supplemented with calcium compared to that in subjects with normal calcium intakes (Berlin & Björkhem, 1988), whereas 2 other RCTs showed no effect of additional calcium on serum 25(OH)D in healthy adults (Goussous *et al*, 2005; McCullough *et al*, 2009). However, caution is needed because all three RCTs had design characteristics that may have affected the interpretation of their findings in relation to vitamin D DRI/DRVs. Data from our recent 15-wk winter-based, randomized, placebo-controlled, double-blind vitamin D_3 intervention study in older adults, aged >50 y (n = 125), who were stratified according to calcium intakes [moderate-low (<700 mg/d) or high (>1000 mg/d) intake], showed responses in serum 25(OH)D concentrations throughout winter as well as indexes of vitamin D activation and catabolism were similar irrespective of whether the older adults had relatively low or high habitual calcium intakes (Cashman *et al*, 2014). Not only do these findings suggest that recent vitamin D DRI/DRV will cover the vitamin D needs of even those individuals who have inadequate calcium intakes (Cashman *et al*, 2014), it also strengthens the case for consideration of widening the range of foods for fortification or bio-fortification with vitamin D beyond those that are also a source of calcium.

2.6 Vitamin D biofortified foods

While traditional fortification practices in which exogenous vitamin D is added to dairy and other foodstuffs will continue to be an important approach for increasing vitamin D in the food supply, the use of vitamin D 'biofortified' [also referred to as 'bio-addition' (Calvo & Whiting, 2013)] foods also merits serious attention (Cashman, 2015; Cashman & Kiely, 2016). In this approach, the animal produce (such as, for example, cultured fish, beef, pork, lamb, chicken, and eggs) could have increased vitamin D and/or 25-hydroxyvitamin D contents by virtue of addition of vitamin D and/or 25-hydroxyvitamin D (where permissible) to the livestock feeds. In bio-fortification, the vitamin D compounds in the resulting foodstuffs from the animals will be incorporated in a manner similar to native vitamin D and unlike in traditional fortification will be under some degree of biological regulatory control mechanisms in the animal. In this regard, it is possible that bio-fortification may hold more consumer appeal in some cases. An important concept in relation to vitamin D bio-fortification is that it can increase the 'total vitamin D activity' of the biofortified foods. Total vitamin D activity is used in several of the food composition tables (Saxholt *et al*, 2008; Zurich, 2010), including the UK McCance & Widdowson tables (FSA, 2008), and accounts not only for the vitamin D content of the food but also the content of the key metabolite in the food, 25-hydroxyvitamin D (which is usually multiplied by a factor of 5). In an RCT setting, consumption of orally consumed synthetic 25-hydroxyvitamin D_3 has been shown to be 5 times more effective than an equivalent amount of vitamin D_3 at improving serum 25(OH)D concentrations of older adults in winter (Cashman *et al*, 2012). Improving the total vitamin D activity (i.e., content of vitamin D_3 plus 25-hydroxyvitamin D × 5) of these foods may be of consequence not only to the population as a whole, but in particular, for low or non-consumers of vitamin D-fortified dairy products and also ethnic sub-groups. For example, in the Irish adult diet, the meat, fish, egg food groups, even without bio-fortification, collectively contribute ~40% to the mean daily intake of vitamin D (Black *et al*, 2015). Van Horn *et al*. (2011) reported that in US adolescent girlsthe meat and bean food group contributed ~4% to the mean daily intake of vitamin D in white girls, but contributed 26% to the mean daily intake of vitamin D in African American girls. The possibility of enhancing the vitamin D activity of meat and other relevant foods further by bio-fortification and its potential impact on population and ethnic subgroup intake estimates is of significant public health nutrition relevance.

The most researched vitamin D biofortified food to date is, without doubt, eggs. In the last 15 years, there have been several reports illustrating that the vitamin D_3 content of eggs can be significantly increased by the greater addition of vitamin D_3 to the feed of hens (Mattila *et al*,

1999; 2003; 2004; 2001; Yao *et al*, 2013; Browning & Cowieson, 2014; Hayes *et al*, 2016), however, several of these studies (Mattila *et al*, 2003; 2004; 2011; Yao *et al*, 2013; Browning & Cowieson, 2014) used levels of inclusion above the upper allowable level for feeds in Europe [3000 IU/kg diet (EC) No. 1831/2003] (see **Figure 1**). Addition of commercially available 25-hydroxyvitamin D_3 to the diet of hens has also been shown to increase egg 25-hydroxyvitamin D_3 content (Mattila *et al*, 2011; Browning & Cowieson, 2014; Hayes *et al*, 2016), albeit two studies (Matilla *et al*, 2011; Browning & Cowieson, 2014) used 25-hydroxyvitamin D_3 at levels above the upper allowable level [0.080 mg/kg diet (EFSA FEEDAP panel, 2005)]. Many of these studies were predominantly focused on the effect that higher levels of vitamin D compounds in animal feeds may have on the welfare of the animal itself or the quality of their produce (e.g. egg shell strength, laying rate) rather than its impact on the vitamin D activity of the resulting eggs and their potential to improve the vitamin D status of humans. In terms of producing eggs that would be acceptable for human consumption, we have recently demonstrated that additional vitamin D and/or 25-hydroxyvitamin D_3 at levels adhering to the maximum allowable EU regulation [(EC) No. 1831/2004; EFSA FEEDAP panel, 2005], resulted in eggs with increased vitamin D activity (providing ~5 μg/egg), and, importantly, no deterioration of consumer acceptability of the biofortified eggs compared to usual eggs (Hayes *et al*, 2016). Thus, such vitamin D-biofortified eggs could supply half the EAR for vitamin D.

As mentioned above, evidence of effectiveness of food fortification approaches from RCTs, which evaluate their impact on reducing the prevalence of vitamin D deficiency in the populations studied, is a key priority in terms of the evidence-base. Importantly, in that context, we have also very recently shown in a winter-based RCT of older adults (n=55) that weekly consumption of 7 vitamin D-biofortified eggs, produced by hens provided with feed containing either vitamin D_3 or 25-hydroxyvitamin D_3 at the allowable maximum content, prevented the typical decline in serum 25(OH)D concentration during winter and any incidence of vitamin D deficiency (Hayes *et al*, 2016). The control group in the study, who were requested to consume weekly up to a maximum of 2 commercially available eggs, had a significant decline in serum 25(OH)D over the 8 weeks of winter, and 22% had vitamin D deficiency (serum 25(OH)D <25 nmol/L) at endpoint (Hayes *et al*, 2016). From a dietary guideline perspective, the general population can include up to seven eggs a week in their diet (Krauss *et al*, 2000), and our RCT showed no difference in serum total cholesterol among control and vitamin D-biofortified egg groups (Hayes *et al*, 2016).

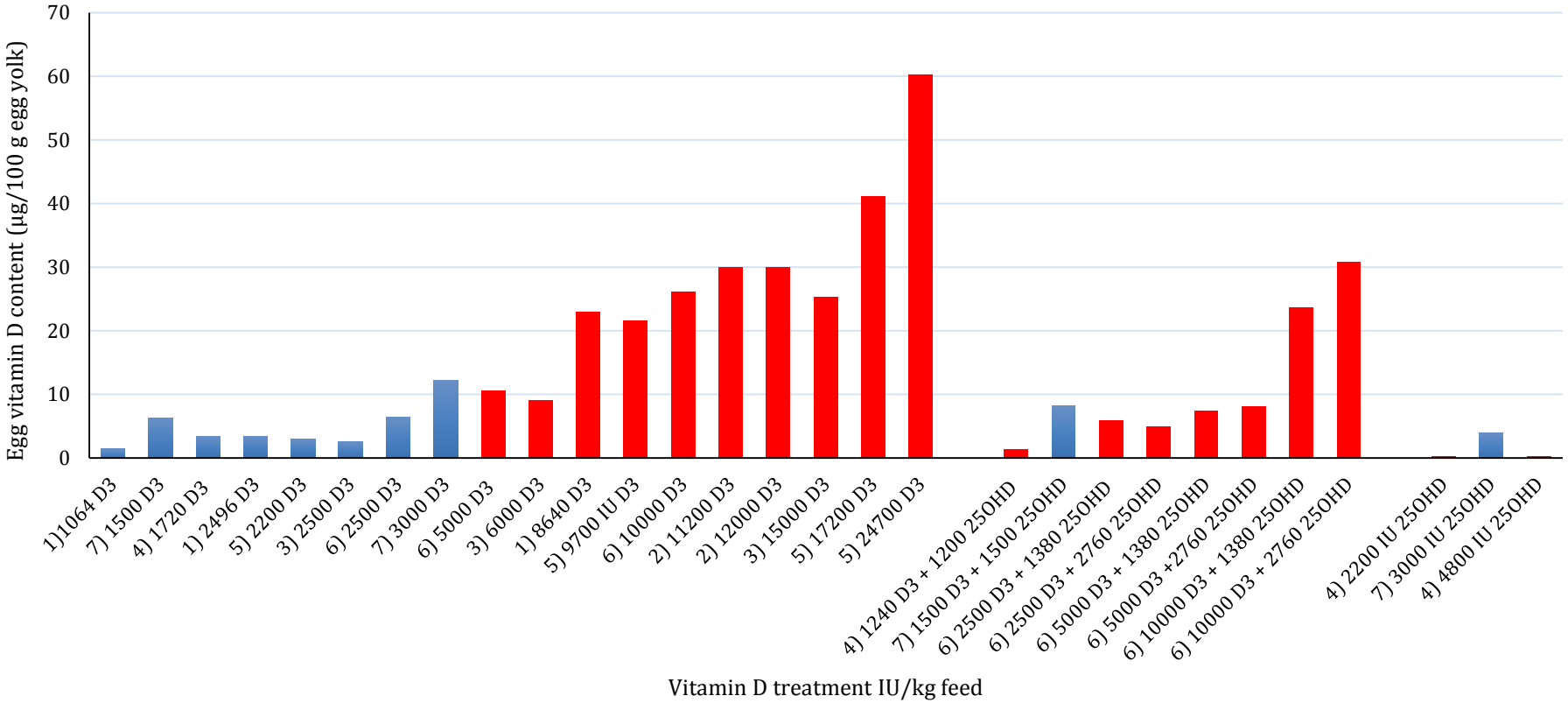

Figure 1. The achieved vitamin D_3 content of egg yolk (µg/100g) reported from published studies on the effect of different vitamin D and 25-hydroxyvitamin D supplementation rates in laying hen feeds. The first number in parenthesis on the X axis denotes the study from which these data are derived as follows: 1) Mattila *et al*., 1999; 2) Mattila *et al*., 2003; 3) Mattila *et al*., 2004; 4) Mattila *et al*., 2011; 5) Yao *et al*., 2013; 6) Browning & Cowieson, 2014; 7) Hayes *et al*., 2016.

In a similar fashion, the potential of bio-fortification of other animal-derived foods (such as beef, pork, and cultured fish) with vitamin D compounds is currently under investigation in a number of animal feeding trials in both an EU Framework 7 (FP7) collaborative project called 'ODIN; Food-based solutions for Optimal vitamin D Nutrition and health through the life cycle' (www.ODIN-vitD.eu) and an Irish nationally funded project ('Enhance*D* Meats'; http://www.ucc.ie/en/vitamind/history/ongoing/enhancedmeats). Some of these vitamin D biofortified foods have also been tested in a winter-based RCT, within the ODIN project, in terms of evaluating their potential at raising serum 25(OH)D in Danish white and South Asian women at high risk of vitamin D deficiency. The results of this research will become available over the next 12 months. Following the demonstration of efficacy of these vitamin D biofortified foods on response of serum 25(OH)D in RCT settings, as mentioned above, dietary modelling analysis based on data from nationally representative dietary surveys within ODIN will provide *in silico* projections of how these food interventions may impact on the degree of vitamin D intake inadequacy in the population.

Finally, bio-fortification with vitamin D could also embrace the practice of UVB-irradiation of mushrooms and baker's yeast, which has been shown to stimulate their endogenous vitamin D_2 content. These foods may be a useful strategy to increase vitamin D intakes for vegetarians or those who do not consume meat or animal-derived foods for cultural reasons, but also for the wider population, particularly in the case of bread, as mentioned above. From a technological perspective, these UV irradiated foods can be produced with anything from low to very high levels of vitamin D_2. However, in terms of RCT-based evidence of effectiveness, a recent 8-week RCT within the ODIN project (Itkonen *et al*, 2016), investigated the bioavailability of D_2 from UV-irradiated yeast present in bread in healthy 20-37-year-old women (n=33) in Helsinki (60°N) during winter. Four study groups were given different study products, either a placebo pill and regular bread (providing 0 µg vitamin D_2 or D_3 per day); a vitamin D_2 supplement and regular bread (providing 25 µg vitamin D_2 per day); a vitamin D_3 supplement and regular bread (providing 25 µg vitamin D_3/d); or a placebo pill and vitamin D_2-fortified bread (made with UV yeast) (providing 25 µg D_2/d). Serum 25(OH)D concentration was measured at baseline, midpoint (week 4) and endpoint (week 8). The study showed that consumption of the UV yeast-vitamin D_2-fortified bread did not affect serum 25(OH)D concentration, unlike the increases seen in those receiving supplemental vitamin D together with regular bread (Itkonen *et al*, 2016). This was despite the fact that the bread, even following the baking process, was analysed and shown to actually contain the 25 µg vitamin D_2. Thus, these new data suggest that vitamin D_2 from UV-irradiated yeast in bread, despite being present post-baking, was not bioavailable in humans.

The RCT data demonstrating that the vitamin D_2 in UV-treated mushrooms can increase vitamin D status of consumers have been quite mixed, and accordingly, we recently undertook a systematic review and meta-analysis of the impact of UV-exposed mushrooms on serum 25(OH)D response in these RCTs (Cashman *et al*, 2016b), again within the ODIN project. Our structured search yielded 6 RCTs meeting our inclusion criteria, and a meta-analysis of these 6 RCTs showed serum 25(OH)D was not significantly increased (P=0.12) by UV-exposed mushrooms, but there was high heterogeneity (I^2=87%). Including only the 3 European-based RCTs [mean baseline 25(OH)D, 38.6 nmol/L], serum 25(OH)D was increased significantly by UV-exposed mushrooms, whereas there was no significant effect in the 3 US-based RCTs [P=0.83; mean baseline 25(OH)D: 81.5 nmol/L] (Cashman *et al*, 2016b). Analysis of serum 25(OH)D_2 and serum 25(OH)D_3 (n=5 RCTs) revealed a statistically significant increase and decrease after supplementation with UV-exposed mushrooms (weighted mean differences of 20.6 nmol/L and -13.3 nmol/L, respectively: $P \leq 0.001$) (Cashman *et al*, 2016b). Thus, consumption of UV-exposed mushrooms may increase serum 25(OH)D when baseline vitamin D status is low via an increase in 25(OH)D_2 and despite a concomitant but relatively smaller reduction in 25(OH)D_3. However, when baseline vitamin D status is high, the mean increase in 25(OH)D_2 and a relatively similar reduction in 25(OH)D_3 explain the lack of effect on serum 25(OH)D.

Artificial UV light technology has also recently been shown to increase the vitamin D_3 content of exposed milk during processing to enhance shelf-life, a process approved by the European Food Safety Authority (EFSA, NDA panel, 2016). Furthermore, UVB light exposure of animals has been shown to improve the vitamin D activity of animal-based foods such as eggs (Kühn *et al*, 2015) and pork (Burild *et al*, 2015).

2.7 Conclusion

While current DRI/DRV for vitamin D are essential public health policy instruments in terms of promoting adequate vitamin D status in the population and prevention of vitamin D deficiency, the present review highlights how even the most recent DRV in Europe differ widely. Notwithstanding this, there is little doubt, irrespective of which set of vitamin D recommendations you choose, that current European population intakes of vitamin D fall well short of these targets. Increasing vitamin D intakes across the population distribution is important from a public health perspective to reduce the high degree of inadequacy of vitamin D intake in Europe and elsewhere, which contribute to prevalence of vitamin D deficiency. Fortification, including bio-fortification, of a wider range of foods, which accommodate diversity, is likely to have the potential to increase vitamin D intakes across the population

distribution and in so doing minimize the prevalence of vitamin D deficiency. Vitamin D biofortified eggs are a good example of one of these novel food-based solutions, which together with other vitamin D-containing foods, can play a role in tackling low vitamin D intakes. The development of vitamin D biofortified eggs is underpinned by a number of hen feeding trials which have examined different vitamin D compounds and doses, and the testing of the resulting egg not only in terms of vitamin D content but also egg quality characteristics and consumer acceptability, but also of key importance, evidence of efficacy in an RCT setting. This exemplifies the role research has had, and continues to have, in terms of developing food-based solutions and tackling vitamin D deficiency.

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Chapter 3

Impact of calcium intake on dietary vitamin D requirements

The core element of the work in this chapter has been published in the following:

Final Project Report to the UK Department of Health. *Inter-relationship between vitamin D requirements for the UK population and calcium intake: does low dietary calcium increase vitamin D requirement*? PRP Reference Number: 024/0049.http://www.prpccf.org.uk/PRPFiles%5CPRP_funded_projects_Summary_final_report_June_2016.pdf

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3.1 Introduction

Calcium and vitamin D are metabolically inter-related to serve endocrine functions within the body. Vitamin D, as the active metabolite (1,25-dihydroxyvitamin D [1,25$(OH)_2$D]), forms part of the endocrine system that maintains serum calcium concentrations within the range necessary for proper regulation of bone mineralisation (DeLuca, 2008), neuromuscular function, vasodilation, nerve transmission and hormonal secretion (Holick, 2011). Interactions between vitamin D and calcium have implications for the regulation of serum 25-hydroxyvitamin D [25(OH)D] concentrations and its catabolism and, consequently, for the dietary vitamin D requirement. For example, if dietary calcium intake remains habitually low, and serum calcium concentrations decrease, the compensatory metabolic response is the activation of 1,25$(OH)_2$D from 25(OH)D (via an increase in circulating parathyroid hormone [PTH] concentration), which normalises serum calcium concentrations. There have been animal data that suggested that the plasma half-life of serum 25(OH)D will be reduced when dietary calcium intake is low, accompanied by an increased hepatic conversion of 25(OH)D to metabolites, which are then excreted in bile (Clements *et al*, 1987).

Mechanistically, the increased synthesis of 1,25$(OH)_2$D during times of low calcium induces the synthesis of the 24-hydroxylase enzyme, which converts serum 25(OH)D to 24,25 dihydroxyvitamin D [24,25$(OH)_2$D]. This metabolite is then further catabolised in the body, and the conversion of 25(OH)D to 24,25$(OH)_2$D is suggested to be the first step in a 5-step, vitamin D-inducible pathway to water soluble truncated degradation products (Jones, 2013). However, older animal data suggests that 24,25$(OH)_2$D (and its metabolite 1,24,25 trihydroxyvitamin D) may stimulate intestinal calcium absorption and bone calcium mobilization (Boyle *et al*, 1971; Holick *et al*, 1973), suggesting a biological activity in addition to a role in the degradation of vitamin D. Clements *et al*, (1992) showed that the elimination half-time of radio-labelled 25(OH)D administered intravenously was significantly shortened by oral 1,25$(OH)_2$D treatment. Further evidence from children with calcium responsive rickets suggest that a low calcium intake induced a higher rate of serum 25(OH)D catabolism (Pettifor, 1991).

While such perturbations may confound the estimation of the true requirement for vitamin D, this has received relatively little research attention to date in healthy humans. Of particular note, the Institute of Medicine (IOM)'s Dietary Reference Intakes (DRI) report on calcium and vitamin D recently highlighted that the elucidation of the inter-relationships

between calcium and vitamin D, and the examination of the influence of calcium intake on the regulation of vitamin D activation and catabolism are key information gaps (IOM, 2011). Even within the relatively limited data that does exist, there is ambiguity. Calcium has been reported as a significant determinant of serum 25(OH)D in some (van der Wielen *et al*, 1995; Kinyamu *et al*, 1998; Hill *et al*, 2004; Andersen *et al*, 2005), but not all observational studies (Andersen *et al*, 2007; Hill *et al*, 2008). One randomised control trial (RCT) in healthy men reported a large increase in serum 25(OH)D in those supplemented with calcium compared to those on their normal calcium intake (Berlin & Björkhem, 1988), while two other RCTs found no effect of additional calcium on serum 25(OH)D in healthy adults (Goussous *et al*, 2005; McCullough *et al*, 2009). However, caution is needed as all three RCTs had design characteristics that may impact on the interpretation of their findings in relation to vitamin D DRIs. For example, vitamin D DRIs have been established for winter-time (IOM, 2011) when serum 25(OH)D concentrations naturally decline, a design consideration which none of the three RCTs addressed, and adequacy of serum 25(OH)D may influence $1,25(OH)_2D$ (Schoenmakers *et al*, 2010; Lagunova *et al*, 2011) which, in turn, influences 24-hydroxylase.

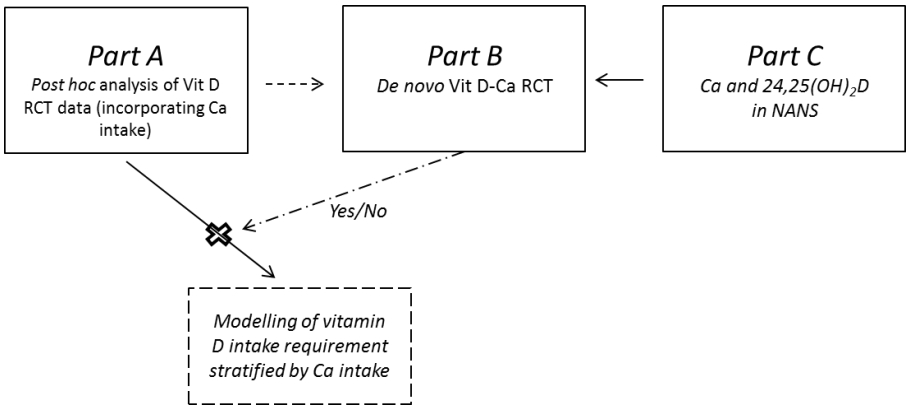
3.1.1 Aims of the present research

The overall aim of the current research was to assess if different levels of habitual calcium intakes influence dietary vitamin D requirements using a three-pronged approach. Firstly, *post-hoc* analysis of existing individual participant data from four placebo-controlled, dose-related vitamin D RCTs in children, adults and the elderly (Cashman *et al*, 2008; Cashman *et al*, 2009; Cashman *et al*, 2011a) was conducted to mathematically model the impact of calcium intake on the vitamin D intake-status relationship [will be referred to as ***Part A***]. This data, in addition to published data from several winter-based vitamin D RCTs (conducted in study locations at >50°N) (Cashman *et al*, 2011b), was also used in a meta-regression approach to further explore the impact of calcium intake on response of 25(OH)D to vitamin D, as well as to inform the design of a *de novo* vitamin D intervention RCT in apparently healthy older adults (>50 years). This winter-based randomised, placebo-controlled, single-dose vitamin D RCT in older adults stratified by calcium intake (<EU Estimated Average Requirement (EAR) [550 mg/d] and >US Recommended Dietary Allowance (RDA) [1000 mg/d] aimed to provide new data on the impact of different levels of calcium intake, ranging from low-moderate to high, on serum 25(OH)D concentrations achieved following increased vitamin D intake [***Part B***]. The effects of these two different levels of calcium intake on indices of vitamin D activation and catabolism, as explanatory variables, during winter-time in the older adults were also determined. The Part A models

would be used to predict the dietary requirement for vitamin D at low-moderate and higher calcium intakes, if the interaction is confirmed in the *de novo* RCT in Part B.

Lastly, to augment the findings of Part A and B, we assessed the impact of low and high calcium intakes in the absence and presence of low and high serum 25(OH)D status on serum $24,25(OH)_2D_3$, using data and bio-banked serum samples from a subset of adults from the representative National Adult Nutrition Survey (NANS) in Ireland (Irish Universities Nutrition Alliance [IUNA], 2011) [***Part C***].

3.1.2 Overview of research approach



Due to the stepwise nature of our approach, where Part A informs Part B and *vice versa*, and Part C may help us to interpret some of the findings of Part B, the *Methods*, *Results* and associated *Discussion* are presented for the three parts separately, and then the findings will be discussed collectively in the *Concluding discussion and remarks* sections of this Chapter.

3.2 Part A – *Post hoc* analysis of existing vitamin D RCT data

3.2.1 Specific objectives

- To perform *post hoc* analysis using existing individual dietary and serum 25(OH)D data from four placebo-controlled, dose-related vitamin D RCTs in children, adults and elderly to assess the impact of levels of calcium intake on the dietary requirement for vitamin D (i.e., the intake needed to maintain serum 25(OH)D above selected thresholds for the majority of individuals).

- To use existing published vitamin D RCT data [(as used recently in meta-regression analysis by the IOM and ourselves (Cashman *et al*, 2011b)] to assess the possible interaction between calcium intake and the response of serum 25(OH)D to vitamin D intake using a meta-regression approach, which may provide for a more representative response as it uses data from several RCT within the model.

3.2.2 Tasks within Part A and associated methodology

Task 1. Mathematical modelling the impact of calcium intake on the vitamin D intake-status relationship.

The present analyses used more complex versions of our previous models (Cashman *et al*., 2008, 2009, 2011a) such that they included calcium intake, both as a continuous variable, as well as a categorical variable (</>EU EAR (550 mg/d), or UK Reference Nutrient Intake [RNI] (700 mg/d), or US EAR/RDA (800/1000 mg/d, dependent on age) to test for an interaction with dietary vitamin D requirement.

Task 2. Within treatment group response of serum 25(OH)D to vitamin D intake at different levels of calcium intake.

Some RCT suggest that calcium intake (as supplemental calcium) modifies the response of serum 25(OH)D to vitamin D, but the effect of habitual calcium intake has not been explored. Whether the achieved mean serum 25(OH)D within the four discrete dose groups (0,5,10,15 μg vitamin D_3/d) in the two adult/older adult RCTs (and three doses [0,5,10 μg vitamin D_3/d] in 11 year-old girls) differs at the end of 5-months intervention following stratification by habitual calcium intake using several cut-offs (outlined in Task 1), and after accounting for possible confounding factors (age, sex, dietary vitamin D, smoking, and BMI), was explored. In addition, for comparative purposes we did this analysis

comparing those with habitual calcium intakes <550 mg/d (and <700 mg/d) versus >1000 mg/d.

Task 3. Update of database of vitamin D RCTs and meta-regression analyses.

As an additional approach, the published group mean data from ~15 winter-based vitamin D RCTs conducted at latitudes >50^{o}N [identified by the IOM and ourselves (Cashman *et al*., 2011b)] were used to investigate a possible interaction between mean group calcium intake and response of serum 25(OH)D to vitamin D intake in meta-regression models which would be cognisant of the vitamin D intake range appropriate for the UK and Ireland. While such group mean data were not from UK/Ireland-only based RCTs, it represented a latitude range of 51-63^{o}N covering from South of England to Scotland. To undertake this task, our database of relevant data from published vitamin D RCT (Cashman *et al*., 2011b) was re-visited to include data on calcium intake, and was also updated for any appropriate vitamin D RCT published between February 2011 and March 2013. Following this data collection, meta-regression analyses was undertaken to test for a possible interaction between calcium intake and vitamin D intake-status relationship (i.e., an interaction between the predicted response of serum 25(OH)D to total vitamin D intake and habitual calcium intake).

Statistical analysis of the data was conducted using SPSS© for Windows™ Version 22.0 (SPSS, Inc., Chicago, IL, USA).

3.2.3 Results of Part A

Task 1. Mathematical modelling of the impact of calcium intake on the vitamin D intake-status relationship.

The regression models used in previous vitamin D RCTs by the *Vitamin D Research Group* (Cashman *et al*., 2008, 2009, 2011a), which aimed to establish the dietary vitamin D requirement, were performed again but this time included habitual calcium intake as a new potential determinant. This analysis showed that category of habitual calcium intake (< and ≥ 550 mg/d, < and ≥ 700 mg/d, < and ≥ 800 mg/d and < and ≥ 1000 mg/d; corresponding to the EU EAR, UK RNI, US EAR and US RDA values, respectively) was not a significant predictor of winter serum 25(OH)D concentration and did not interact with total vitamin D intake in 11 year-old girls, 20-40 year-old healthy adults, or 64+ year-old older adults (P>0.1, in all cases). Likewise, the lack of significance of habitual calcium intake in the models remained the case (P>0.3 in all cases) when it was included as a continuous variable (mg/d) rather than as a categorical above or below the threshold.

Task 2. Within treatment group response of serum 25(OH)D to vitamin D intake at different levels of calcium intake.

3.2.3.1 Baseline serum 25(OH)D concentration in those above and below thresholds of habitual calcium intake in post-hoc analysis

Following stratification of individual data points within each treatment group according to their habitual calcium intake being above or below four different cut-off points i.e. 550, 700, 800, and 1000 mg/d, there was no significant difference in baseline serum 25(OH)D concentrations in 20-40 year old healthy adults (**Table 1**) or older adults, 64+ years (**Table 2**), with the exception of those older adults with habitual calcium intakes above or below 700 mg/d where a significant difference did exist (P ≤0.05); (Median [IQR; n] serum 25(OH)D of 46.2 [39.2, 59.7; 58] versus 59.5 [43.3, 75.0; 142] nmol/L for < and ≥ 700 mg/d, respectively; Table 2). However, following adjustment within the model for sex, age, study centre, smoking and BMI, this statistical difference no longer existed (P=0.189).

Baseline serum 25(OH)D concentrations of 11-year-old girls (n=155) who participated in a vitamin D intervention trial stratified according to several cut-off points for habitual calcium intake are shown in **Table 3**. There were significant differences in baseline serum 25(OH)D concentrations between groups < or ≥ 700 mg Ca/d (P=0.028) and < or 800 mg/d ≥ 800 mg Ca/d (P=0.026).

Table 1. Baseline serum 25-hydroxyvitamin D [25(OH)D] in 20-40 year-old healthy adults who participated in a vitamin D RCT (Cashman *et al*., 2008), stratified by thresholds of habitual calcium intake

Calcium intake threshold (mg/d)	Baseline serum 25(OH)D (nmol/L)*					
	Below threshold			Above threshold		
	Median	IQR	*n*	Median	IQR	*n*
550	65.9	45.6-86.2	22	70.9	54.4-94.9	192
700	69.9	57.0-89.6	50	70.8	53.3-91.3	164
800	67.3	53.2-88.7	70	72.6	54.4-93.0	144
1000	65.8	53.8-86.2	110	76.0	53.4-98.0	104

RCT, Randomised Control Trial; IQR, interquartile range.
*No significant difference were observed; *via* unpaired *t*-tests, $P>0.05$ in all cases

Table 2. Baseline serum 25-hydroxyvitamin D [25(OH)D] concentrations in healthy older adults (64+ years) who participated in a vitamin D RCT (Cashman *et al*., 2009), stratified by thresholds of habitual calcium intake.

Calcium intake threshold (mg/d)	Baseline serum 25(OH)D (nmol/L)					
	Below threshold			Above threshold		
	Median	IQR	*n*	Median	IQR	*n*
550	44.2	39.3-79.8	19	55.9	41.7-72.1	181
700	46.2	39.2-59.7	58	59.5*	43.3-75.0	142
800	48.2	39.3-67.8	83	59.4	44.0-75.0	117
1000	53.6	41.6-71.5	123	57.9	40.6-73.8	77

RCT, Randomised Control Trial; IQR, interquartile range.
*Significantly different from below threshold value; *via* unpaired *t*-tests, $P<0.05$

Table 3. Baseline serum 25-hydroxyvitamin D [25(OH)D] concentrations in 11 year-old healthy girls who participated in a vitamin D RCT (Cashman *et al*., 2011a), stratified by thresholds of habitual calcium intake.

Calcium intake threshold (mg/d)	Baseline serum 25(OH)D (nmol/L)					
	Below threshold			Above threshold		
	Mean	SD	*n*	Mean	SD	*n*
550	54.0	16.7	25	57.4	16.7	130
700	52.8	14.7	41	53.3*	12.9	114
800	53.2	13.9	48	58.5*	13.2	107
1000	53.8	15.2	68	58.6¶	17.7	87

RCT, Randomised Control Trial; IQR, interquartile range.
*Significantly different from below threshold value; *via* unpaired *t*-tests, $P<0.05$.
¶Trend towards being different from below threshold value; *via* unpaired *t*-tests, $P=0.064$.

A borderline difference existed for < or ≥ 1000 mg/d (P= 0.064) but no such difference was present between groups < or ≥ 550 mg/d.

Adjustment for study centre and BMI in these models had no effect on these outcomes in any of the groups. The significant differences in the 11 year old girls with habitual calcium intakes < or ≥ 700, 800 and 1000 mg/d were strengthened after controlling for the above confounding factors.

3.2.3.2 Response of serum 25(OH)D concentrations to intervention in all groups

The response of serum 25(OH)D concentrations to dose-related vitamin D supplementation in winter-time during these trials have been stratified using the aforementioned thresholds for habitual calcium intake and are outlined in **Tables 4-6**. Significant differences existed between placebo and intervention groups, as expected, in the response of serum 25(OH)D concentrations to supplementation with vitamin D (P<0.0001, in all cases).

The change in winter serum 25(OH)D concentrations from pre- to post- intervention in 11 year-old girls that had been stratified by habitual calcium intakes of < or ≥ 550 (**Table 4a**), 700 (**Table 4b**), 800 (**Table 4c**), and 1000 mg/d (**Table 4d**) were compared separately in each of the treatment groups (Placebo, 5 µg, 10 µg vitamin D/d). No significant differences were observed (P > 0.05, in all cases). Similarly, following stratification of the intervention groups as above, the change in winter serum 25(OH)D concentrations from pre- to post- intervention in each of the 4 treatment groups (Placebo, 5 µg, 10 µg, 15 µg) were examined independently in healthy 20-40 year olds (**Tables 5a-d**) and no significant differences were observed (P≥ 0.1, in all cases).

Pre- and post-intervention serum 25(OH)D concentrations in 64+ year olds stratified by a habitual calcium intake threshold of 550 mg/d across vitamin D treatment groups (Placebo, 10 vitamin D µg/d, 15 vitamin D µg/d) are shown in **Table 6a**. Data are not included for the 5 µg vitamin D/d treatment group as upon stratification by 550 mg Ca/d, there were only 2 subjects with a habitual calcium intake of less than 550 mg/d. The change in serum 25(OH)D concentration following intervention with placebo or vitamin D is also shown. There were no significant differences in the change of serum 25(OH)D concentrations between groups stratified by 550 mg Ca/d in any vitamin D treatment group (P>0.05, in all cases).

Table 4a. Serum 25-hydroxyvitamin D (25(OH)D) concentration among vitamin D treatment groups pre- and post-intervention and its change in 11 year-old girls, stratified by habitual calcium intake above or below a selected threshold of 550 mg/d[1].

Treatment Groups	Placebo	5 µg vitamin D/d	10 µg vitamin D/d
Habitual calcium intake <550mg Ca/d			
n	9	9	7
Serum 25(OH)D (nmol/L):			
Pre-intervention[2]	47.5 (11.3)	60.5 (16.4)	54.1 (21.3)
Post-intervention[3]	20.9 (13.4)	44.6 (4.9)	59.0 (9.9)
Change	-26.6 (19.2)	-15.9 (14.4)	4.9 (14.7)
Habitual calcium intake >550mg Ca/d			
n	46	46	45
Serum 25(OH)D (nmol/L):			
Pre-intervention[2]	54.1 (18.7)	54.3 (16.3)	57.4 (15.1)
Post-intervention[3]	28.9 (13.5)	42.1 (18.2)	53.6 (19.8)
Change	-25.2 (18.0)	-12.2 (17.4	-3.8 (23.9)
P-value[4]	0.081	0.054	0.378

[1]Values represent means (SD)

[2] All baseline blood samples of girls (a subset of those in the original 12-month intervention trials) were taken between September and October, 2001 or 2002

[3] All endpoint blood samples of girls (a subset of those in the original 12-month intervention trials) were taken between March and April, 2001 or 2002

[4] Unpaired *t*-test of the change from pre- to post- intervention serum 25(OH)D concentration between those below and above the habitual calcium intake.

Table 4b. Serum 25-hydroxyvitamin D (25(OH)D) concentration among vitamin D treatment groups pre- and post-intervention and its change in 11 year-old girls, stratified by habitual calcium intake above or below a selected threshold of 700 mg/d[1].

Treatment Groups	Placebo	5 µg vitamin D/d	10 µg vitamin D/d
Habitual calcium intake <700mg Ca/d			
n	15	16	10
Serum 25(OH)D (nmol/L):			
Pre-intervention[2]	49.1 (9.9)	55.3 (16.2)	54.4 (18.1)
Post-intervention[3]	23.9 (11.6)	46.6 (6.4)	52.1 (20.3)
Change	-25.3 (15.3)	-8.8 (15.3)	-2.3 (25.9)
Habitual calcium intake >700mg Ca/d			
n	38	39	42
Serum 25(OH)D (nmol/L):			
Pre-intervention[2]	54.5 (20.0)	55.3 (16.6)	57.5 (15.4)
Post-intervention[3]	29.0 (14.3)	40.8 (19.3)	54.8 (18.8)
Change	-25.5 (19.3)	-14.5 (17.4)	-2.7 (22.6)
P-value[4]	0.255	0.924	0.303

[1]Values represent means (SD)

[2] All baseline blood samples of girls (a subset of those in the original 12-month intervention trials) were taken between September and October, 2001 or 2002

[3] All endpoint blood samples of girls (a subset of those in the original 12-month intervention trials) were taken between March and April, 2001 or 2002

[4] Unpaired *t*-test of the change from pre- to post- intervention serum 25(OH)D concentration between those below and above the habitual calcium intake.

Table 4c. Serum 25-hydroxyvitamin D (25(OH)D) concentration among vitamin D treatment groups pre- and post-intervention and its change in 11 year-old girls, stratified by habitual calcium intake above or below a selected threshold of 800 mg/d[1].

Treatment Groups	Placebo	5 µg vitamin D/d	10 µg vitamin D/d
Habitual calcium intake <800mg Ca/d			
n	16	20	12
Serum 25(OH)D (nmol/L):			
Pre-intervention[2]	49.1 (9.6)	55.6 (15.1)	54.7 (16.4)
Post-intervention[3]	23.8 (11.2)	45.5 (7.3)	54.0 (19.1)
Change	-25.3 (14.7)	-10.1 (14.6)	-0.7 (23.8)
Habitual calcium intake >800mg Ca/d			
n	37	35	40
Serum 25(OH)D (nmol/L):			
Pre-intervention[2]	54.6 (20.2)	55.2 (17.2)	57.6 (15.8)
Post-intervention[3]	29.2 (14.4)	40.7 (20.2)	54.4 (19.2)
Change	-25.2 (19.9)	-14.4 (18.1)	-3.2 (23.0)
P-value[4]	0.311	0.476	0.189

[1]Values represent means (SD)

[2] All baseline blood samples of girls (a subset of those in the original 12-month intervention trials) were taken between September and October, 2001 or 2002

[3] All endpoint blood samples of girls (a subset of those in the original 12-month intervention trials) were taken between March and April, 2001 or 2002

[4] Unpaired *t*-test of the change from pre- to post- intervention serum 25(OH)D concentration between those below and above the habitual calcium intake.

Table 4d. Serum 25-hydroxyvitamin D (25(OH)D) concentration among vitamin D treatment groups pre- and post-intervention and its change in 11 year-old girls, stratified by habitual calcium intake above or below a selected threshold of 1000 mg/d[1].

Treatment Groups	Placebo	5 µg vitamin D/d	10 µg vitamin D/d
Habitual calcium intake <1000mg Ca/d			
n	25	27	17
Serum 25(OH)D (nmol/L):			
Pre-intervention[2]	51.2 (12.1)	56.9 (14.1)	52.5 (20.4)
Post-intervention[3]	26.0 (10.2)	47.3 (7.8)	56.3 (16.8)
Change	-25.3 (14.0)	-9.6 (12.7)	3.9 (26.1)
Habitual calcium intake >1000mg Ca/d			
n	28	28	35
Serum 25(OH)D (nmol/L):			
Pre-intervention[2]	54.5 (21.8)	53.8 (18.3)	59.1 (12.8)
Post-intervention[3]	29.0 (16.2)	37.8 (21.4)	53.3 (20.1)
Change	-25.2 (21.3)	-16.0 (19.9)	-5.8 (21.0)
P-value[4]	0.946	0.444	0.217

[1]Values represent means (SD)

[2] All baseline blood samples of girls (a subset of those in the original 12-month intervention trials) were taken between September and October, 2001 or 2002

[3] All endpoint blood samples of girls (a subset of those in the original 12-month intervention trials) were taken between March and April, 2001 or 2002

[4] Unpaired *t*-test of the change from pre- to post- intervention serum 25(OH)D concentration between those below and above the habitual calcium intake.

Table 5a. Serum 25-hydroxyvitamin D (25(OH)D) concentration among vitamin D treament groups pre- and post-intervention and its change in 20-40 year-old adults, stratified by habitual calcium intake above or below a selected threshold of 550 mg/d[1].

Treatment Groups	Placebo	5 µg vitamin D/d	10 µg vitamin D/d	15 µg vitamin D/d
Habitual calcium intake <550mg Ca/d				
n	5	5	6	6
Serum 25(OH)D nmol/L):				
Pre-intervention[2]	61.2 [45.4, 113.7]	53.7 [45.4, 77.4]	64.6 [38.4, 97.3]	85.0 [69.5, 88.7]
Post-intervention[3]	40.0 [30.8, 63.0]	46.6 [45.7, 58.7]	54.6 [31.3, 76.7]	74.5 [61.4, 79.1]
Change	-23.5 [-52.3, -13.4]	-8.2 [-24.3, 6.4]	-8.8 [-38.2,6.2]	-10.5 [-23.0, 5.6]
Habitual calcium intake >550mg Ca/d				
n	51	44	51	46
Serum 25(OH)D nmol/L):				
Pre-intervention[2]	66.5 [59.0, 96.2]	60.0 [50.5, 94.5]	73.1 [56.8, 95.4]	75.5 [55.9, 91.8]
Post-intervention[3]	35.8 [30.7, 48.2]	50.5 [43.9, 61.8]	62.0 [50.0, 68.7]	68.5 [59.1, 85.0]
Change	-26.8 [-48.7, -22.3]	-14.6 [-31.9, -5.4]	-15.7 [-27.8, 3.7]	-9.8 [-25.6, 18.7]
P-value[4]	0.288	0.307	0.929	0.625

[1]Values represent medians [interquartile range] as non-normally distributed

[2]All baseline blood samples taken between 2nd October – 7th November, 2006.

[3]All endpoint blood samples taken between 27th February – 7th April, 2007

[4]Unpaired *t*-test of the change from pre- to post- intervention serum 25(OH)D concentration between those below and above the habitual calcium intake.

Table 5b. Serum 25-hydroxyvitamin D (25(OH)D) concentration among vitamin D treatment groups pre- and post-intervention and its change in 20-40 year-old adults, stratified by habitual calcium intake above or below a selected threshold of 700 mg/d[1].

Treatment Groups	Placebo	5 µg vitamin D/d	10 µg vitamin D/d	15 µg vitamin D/d
Habitual calcium intake <700mg Ca/d				
n	13	10	14	13
Serum 25(OH)D nmol/L):				
Pre-intervention[2]	72.4 [48.8, 110.2]	59.3 [51.8, 75.2]	66.2 [54.8, 111.4]	78.1 [66.7, 89.2]
Post-intervention[3]	41.8 [33.5, 64.3]	46.8 [45.7, 58.3]	62.8 [45.8, 74.7]	75.0 [64.9, 84.6]
Change	-25.4 [-72.5, -15.0]	-12.5 [-18.8, -6.1]	-8.8 [-35.8, 2.6]	-10.5 [-24.7, 15.9]
Habitual calcium intake >700mg Ca/d				
n	43	39	43	39
Serum 25(OH)D nmol/L):				
Pre-intervention[2]	72.4 [56.3, 109.2]	60.1 [50.0, 96.5]	75.8 [56.6, 90.8]	76.0 [54.3, 90.0]
Post-intervention[3]	35.7 [29.7, 47.9]	50.6 [41.9, 62.5]	59.0 [50.0, 68.6]	67.6 [59.1, 84.9]
Change	-26.8 [-45.2, -22.6]	-14.8 [-34.7, -4.6]	-15.7 [-27.8, 3.8]	-11.3 [-25.6, 18.7]
P-value[4]	0.165	0.562	0.982	0.727

[1]Values represent medians [interquartile range] as non-normally distributed

[2]All baseline blood samples taken between 2nd October – 7th November, 2006.

[3]All endpoint blood samples taken between 27th February – 7th April, 2007

[4]Unpaired *t*-test of the change from pre- to post- intervention serum 25(OH)D concentration between those below and above the habitual calcium intake.

Table 5c. Serum 25-hydroxyvitamin D (25(OH)D) concentration among vitamin D treatment groups pre- and post-intervention and its change in 20-40 year-old adults, stratified by habitual calcium intake above or below a selected threshold of 800 mg/d[1].

Treatment Groups	Placebo	5 µg vitamin D/d	10 µg vitamin D/d	15 µg vitamin D/d
Habitual calcium intake <800mg Ca/d				
n	18	18	18	16
Serum 25(OH)D nmol/L):				
Pre-intervention[2]	68.0 [45.5, 100.3]	64.5 [53.8, 82.9]	66.2 [54.8, 111.4]	72.8 [48.7, 87.5]
Post-intervention[3]	40.3 [31.4, 51.0]	47.3[45.4, 58.3]	63.0 [51.5, 74.7]	74.5 [60.2,83.8]
Change	-25.2 [-63.8, -15.9]	-13.3 [-28.9, -8.6]	-5.8 [-26.9, 5.4]	-0.2 [-16.5, 23.4]
Habitual calcium intake >800mg Ca/d				
n	38	31	39	36
Serum 25(OH)D nmol/L):				
Pre-intervention[2]	65.6 [59.1, 90.9]	57.8 [48.8, 96.5]	76.2 [56.6, 90.8]	77.3 [60.7, 96.4]
Post-intervention[3]	35.8 [30.4, 48.1]	50.8 [40.6, 62.5]	57.7 [45.2, 68.6]	67.9 [59.1, 85.0]
Change	-27.8 [-46.0, -22.7]	-14.3 [-32.1, -2.5]	-18.9 [-32.4, 0.4]	-12.9 [-29.0, 12.0]
P-value[4]	0.331	0.812	0.337	0.171

[1]Values represent medians [interquartile range] as non-normally distributed

[2]All baseline blood samples taken between 2nd October – 7th November, 2006.

[3]All endpoint blood samples taken between 27th February – 7th April, 2007

[4]Unpaired *t*-test of the change from pre- to post- intervention serum 25(OH)D concentration between those below and above the habitual calcium intake.

Table 5d. Serum 25-hydroxyvitamin D (25(OH)D) concentration among vitamin D treatment groups pre- and post-intervention and its change in 20-40 year-old adults, stratified by habitual calcium intake above or below a selected threshold of 1000 mg/d[1].

Treatment Groups	Placebo	5 µg vitamin D/d	10 µg vitamin D/d	15 µg vitamin D/d
Habitual calcium intake <1000mg Ca/d				
n	32	26	29	23
Serum 25(OH)D nmol/L):				
Pre-intervention[2]	65.6 [52.8, 92.6]	59.3 [50.2, 78.5]	66.5 [58.6, 89.2]	76.2 [54.3, 88.2]
Post-intervention[3]	37.4 [29.9, 47.9]	46.7 [41.5, 57.6]	62.0 [46.3, 71.6]	75.0 [60.3, 84.9]
Change	-25.8 [-38.7, -21.8]	-11.7 [-24.0, -4.6]	-9.7 [-24.2, 3.8]	-0.7 [-18.2, 21.8]
Habitual calcium intake >1000mg Ca/d				
n	24	23	28	29
Serum 25(OH)D nmol/L):				
Pre-intervention[2]	67.7 [60.1, 102.7]	76.2 [50.0, 99.4]	81.2 [50.7, 101.1]	75.9 [58.4, 88.2]
Post-intervention[3]	36.8 [32.3, 48.6]	53.4 [48.8, 67.4]	59.3 [50.3, 67.8]	67.6 [58.0, 84.5]
Change	-32.6 [-52.2, -22.4]	-15.7 [-37.5, -4.6]	-16.6 [-35.5, -0.1]	-12.6 [-25.6, 11.5]
P-value[4]	0.212	0.596	0.650	0.206

[1]Values represent medians [interquartile range] as non-normally distributed

[2]All baseline blood samples taken between 2nd October – 7th November, 2006.

[3]All endpoint blood samples taken between 27th February – 7th April, 2007

[4]Unpaired *t*-test of the change from pre- to post- intervention serum 25(OH)D concentration between those below and above the habitual calcium intake.

Table 6a. Serum 25-hydroxyvitamin D (25(OH)D) concentration among vitamin D treatment groups pre- and post-intervention and its change in 64+ year-old adults, stratified by habitual calcium intake above or below a selected threshold of 550 mg/d[1*].

Treatment Groups	Placebo	10 µg vitamin D/d	15 µg vitamin D/d
Habitual calcium intake <550mg Ca/d			
n	5	7	6
Serum 25(OH)D nmol/L):			
Pre-intervention[2]	79.8 [48.8, 113.0]	45.9 [33.3, 88.2]	41.8 [34.3, 49.7]
Post-intervention[3]	56.2 [37.9, 62.8]	65.9 [58.6, 84.2]	68.1 [65.1, 79.1]
Change	-23.5 [-62.1, 0.92]	15.4 [2.2, 34.9]	31.7 [24.7, 39.0]
Habitual calcium intake >550mg Ca/d			
n	48	44	44
Serum 25(OH)D nmol/L):			
Pre-intervention[2]	59.0 [39.9, 75.5]	56.9 [43.5, 72.3]	55.1 [38.7, 69.9]
Post-intervention[3]	39.6 [27.4, 54.2]	70.0 [58.2, 81.2]	68.2 [65.1, 95.2]
Change	-14.1 [-22.6, -6.1]	10.6 [-1.0, 19.5]	21.8 [7.0, 32.1]
P-value[4]	0.534	0.180	0.093

[1]Values represent medians [interquartile range] as non-normally distributed

*The 5 µg vitamin D/d group was not included since there was only one subject with a habitual calcium intake , <550 mg Ca/d

[2]All baseline blood samples taken between 28th September – 16th November, 2007.

[3]All endpoint blood samples taken between 21st February and 5th April, 2008.

[4]Unpaired *t*-test of the change from pre- to post- intervention serum 25(OH)D concentration between those below and above the habitual calcium intake.

Pre- and post-intervention serum 25(OH)D concentrations in 64+ year olds stratified by a habitual calcium intake threshold of 700 mg/d across vitamin D treatment groups (Placebo, 5 µg vitamin D/d, 10 vitamin D µg/d, 15 vitamin D µg/d) are presented in **Table 6b**. The change in serum 25(OH)D concentration following intervention with placebo or vitamin D is also shown. There were significant differences in the change of serum 25(OH)D concentrations stratified by 700 mg Ca/d in the 5 µg vitamin D/d (P=0.039) and 10 µg vitamin D/d (P=0.019) treatment groups. There were no significant differences in the change of serum 25(OH)D concentrations between groups stratified by 700 mg Ca/d, in the placebo and 15 µg vitamin D/d treatment groups (P>0.05, in both).

Pre- and post-intervention serum 25(OH)D concentrations in 64+ year olds stratified by a habitual calcium intake threshold of 800 mg/d across vitamin D treatment groups (Placebo, 5 µg vitamin D/d, 10 vitamin D µg/d, 15 vitamin D µg/d) are shown in **Table 6c**. The change in serum 25(OH)D concentration following intervention with placebo or vitamin D is also shown. There was a significant difference in the change of serum 25(OH)D concentrations between groups stratified by 800 mg Ca/d in the 5 µg vitamin D/d treatment group (P=0.009). There were no significant differences in the change of serum 25(OH)D concentrations between treatment groups (P>0.05, in all cases).

Pre- and post-intervention serum 25(OH)D concentrations in 64+ year olds stratified by a habitual calcium intake threshold of 1000 mg/d across vitamin D treatment groups (Placebo, 5 µg vitamin D/d, 10 vitamin D µg/d, 15 vitamin D µg/d) are shown in **Table 6d**. The change in serum 25(OH)D concentration following intervention with placebo or vitamin D is also shown. There was a significant difference in the change of serum 25(OH)D concentrations between groups stratified by 1000 mg Ca/d in the 10 µg vitamin D/d group (P=0.034). There were no significant differences in the change of serum 25(OH)D concentrations between groups stratified by 1000 mg Ca/d in the remaining vitamin D treatment groups (P>0.05, in all cases).

However, further analysis showed that after controlling for possible confounding effects (of sex, age, dietary vitamin D, BMI, smoking), the response of winter serum 25(OH)D does not differ in 64+ year-old adults by level of habitual calcium intake above and below any of the four thresholds (P>0.1 in all cases; data not shown). Additional analysis in which the response of those with habitual calcium intakes <550 mg/d versus >1000 mg/d, unadjusted and adjusted for the confounding effects of sex, age, dietary vitamin D, BMI, smoking, showed no significant differences in the response of winter serum 25(OH)D in the girls, 20-40 year-old adults or 64+ year-old adults, separately (data not shown).

Table 6b. Serum 25-hydroxyvitamin D (25(OH)D) concentration among vitamin D treatment groups pre- and post-intervention and its change in 64+ year-old adults, stratified by habitual calcium intake above or below a selected threshold of 700 mg/d[1].

Treatment Groups	Placebo	5 µg vitamin D/d	10 µg vitamin D/d	15 µg vitamin D/d
Habitual calcium intake <700mg Ca/d				
n	13	9	20	16
Serum 25(OH)D nmol/L):				
Pre-intervention[2]	44.0 [33.7, 69.3]	39.3 [36.3, 50.7]	48.8 [43.0, 65.1]	51.5 [41.2, 62.5]
Post-intervention[3]	34.9 [25.8, 52.9]	49.9 [45.9, 61.0]	67.5 [58.7, 90.7]	78.8 [67.2, 91.8]
Change	-14.2 [-23.7, -2.8]	6.5 [3.1, 17.9]	16.5 [2.8, 30.6]	31.1 [20.6,34.7]
Habitual calcium intake >700mg Ca/d				
n	40	37	31	34
Serum 25(OH)D nmol/L):				
Pre-intervention[2]	63.0 [45.3, 78.5]	62.3 [45.2, 75.0]	61.8 [43.8, 76.3]	52.7 [37.4, 71.2]
Post-intervention[3]	42.7 [28.0, 58.6]	53.7 [45.5, 71.6]	71.5 [58.0, 81.2]	67.2 [60.2, 86.4]
Change	-14.3 [-22.9, -7.9]	-6.5 [-12.5, 12.5]	8.2 [-8.3, 16.6]	21.4 [6.8, 28.3]
P-value[4]	0.864	0.039	0.019	0.115

[1]Values represent medians [interquartile range] as non-normally distributed

[2]All baseline blood samples taken between 28th September – 16th November, 2007.

[3]All endpoint blood samples taken between 21st February and 5th April, 2008.

[4]Unpaired *t*-test of the change from pre- to post- intervention serum 25(OH)D concentration between those below and above the habitual calcium intake.

Table 6c. Serum 25-hydroxyvitamin D (25(OH)D) concentration among vitamin D treatment groups pre- and post-intervention and its change in 64+ year-old adults, stratified by habitual calcium intake above or below a selected threshold of 800 mg/d[1].

Treatment Groups	Placebo	5 µg vitamin D/d	10 µg vitamin D/d	15 µg vitamin D/d
Habitual calcium intake <800mg Ca/d				
n	20	12	27	24
Serum 25(OH)D nmol/L):				
Pre-intervention[2]	57.8 [38.1, 79.7]	41.3 [35.3, 65.3]	51.2 [43.3, 67.8]	47.2 [37.3, 62.5]
Post-intervention[3]	47.4 [26.3, 56.5]	50.6 [45.3, 75.1]	66.9 [56.4, 84.2]	74.0 [60.4, 90.2]
Change	-12.9 [-23.8, -4.3]	6.1 [1.7, 16.4]	14.4 [2.2, 25.3]	24.8 [15.5, 34.1]
Habitual calcium intake >800mg Ca/d				
n	33	34	24	26
Serum 25(OH)D nmol/L):				
Pre-intervention[2]	59.6 [44.6, 75.3]	59.9 [45.3, 74.8]	64.4 [42.2, 78.5]	56.0 [38.3, 71.9]
Post-intervention[3]	39.5 [27.8, 54.5]	53.2 [45.5, 68.9]	72.6 [59.3, 81.1]	69.3 [61.5, 88.7]
Change	-15.6 [-22.8, -11.1]	-6.8 [-13.8, 12.5]	7.7 [-8.8, 18.3]	21.8 [5.4, 31.5]
P-value[4]	0.644	0.009	0.140	0.204

[1]Values represent medians [interquartile range] as non-normally distributed

[2]All baseline blood samples taken between 28th September – 16th November, 2007.

[3]All endpoint blood samples taken between 21st February and 5th April, 2008.

[4]Unpaired *t*-test of the change from pre- to post- intervention serum 25(OH)D concentration between those below and above the habitual calcium intake.

Table 6d. Serum 25-hydroxyvitamin D (25(OH)D) concentration among vitamin D treatment groups pre- and post-intervention and its change in 64+ year-old adults, stratified by habitual calcium intake above or below a selected threshold of 1000 mg/d[1].

Treatment Groups	Placebo	5 µg vitamin D/d	10 µg vitamin D/d	15 µg vitamin D/d
Habitual calcium intake <800mg Ca/d				
n	33	24	35	31
Serum 25(OH)D nmol/L):				
Pre-intervention[2]	58.8 [38.6, 78.7]	51.2 [38.6, 71.2]	52.3 [43.3, 69.6]	52.6 [41.0, 69.6]
Post-intervention[3]	40.3 [27.4, 56.4]	50.4 [45.4, 73.2]	68.1 [58.5, 83.8]	77.7 [62.3, 86.1]
Change	-13.4 [-23.7, -6.5]	3.44 [-9.2, 14.9]	14.4 [1.8, 25.4]	24.1 [9.0, 34.1]
Habitual calcium intake >800mg Ca/d				
n	33	34	24	26
Serum 25(OH)D nmol/L):				
Pre-intervention[2]	61.4 [44.9, 77.7]	58.7 [42.2, 74.8]	64.4 [41.8, 91.0]	55.7 [36.6, 64.1]
Post-intervention[3]	39.5 [27.7, 55.1]	55.3 [45.5, 68.9]	72.6 [58.2, 80.8]	66.8 [60.3, 104.3]
Change	-15.6 [-22.6, -6.1]	-6.8 [-10.9, 12.5]	7.7 [-13.8, 16.1]	23.8 [7.0, 32.9]
P-value[4]	0.723	0.537	0.034	0.718

[1]Values represent medians [interquartile range] as non-normally distributed

[2]All baseline blood samples taken between 28th September – 16th November, 2007.

[3]All endpoint blood samples taken between 21st February and 5th April, 2008.

[4]Unpaired *t*-test of the change from pre- to post- intervention serum 25(OH)D concentration between those below and above the habitual calcium intake

This was also the case when <700 mg/d versus >1000 mg/d calcium intake cut-offs were compared, except for a significant difference in 64+ year olds adults which disappeared when controlled for confounding factors (data not shown).

Task 3. Update of database of vitamin D RCTs and meta-regression analyses.

The database of published vitamin D RCTs performed in winter and at regions of latitude >46^{o}N (or S) was updated using the same search strategy as used in our recent meta-regression (Cashman *et al*., 2011b). The search was from February 2011 to March 2013 so as to update our previous systematic review. The updated search yielded several new vitamin D RCTs but after consideration of inclusion and exclusion criteria we only identified two new vitamin D RCTs that could be included (Cashman *et al*., 2012; Logan *et al*., 2012). Data from these two new RCTs were extracted as well as mean calcium intake data from the RCTs in the original database (Cashman *et al*., 2011b). One of the RCTs in the original database (Smith *et al*., 2009) did not report calcium intake and as the group used in the RCT was multi-ethnic we could not identify a surrogate calcium intake from nationally representative intake data which could be used for this study. Therefore, this RCT was not included in the present analysis. The data acquired was used to test for an interaction between the predicted response of winter serum 25(OH)D to total vitamin D intake and mean habitual calcium intake in a meta-regression model approach as used by us previously (Cashman *et al*., 2011b).

A review of the RCTs to be included in this analysis showed that the number of RCT arms with mean intakes of calcium below 550, 700 or 800 mg/d (as per Task 1 and 2) was extremely limited at 0, 0 and 1, respectively (see **Table 7**). Therefore, as these cut-offs could not be used, we choose to use the median of the reported mean calcium intakes from the 11 RCTs in the updated Vitamin D RCT database as a cut-off. The RCT arms were stratified on the basis of their mean calcium intakes being above or below this median intake of 1092 mg/d. This yielded *n* 16 and *n* 12 RCT arms whose mean habitual calcium intakes were below or above this median intake, respectively (Table 7).

A weighted linear regression analysis (total vitamin D intake versus achieved winter-time serum 25(OH)D) was performed for each of the two sub-groups of RCT arms separately. The relationship between total vitamin D intake versus achieved winter-time serum 25(OH)D in the two subgroups of RCT arms is shown in **Figure 1**. The regression lines cross suggesting that an interaction exists between the response of winter serum 25(OH)D to total vitamin D and dietary calcium category (mean < and ≥ 1092 mg/d), such that those with lower mean calcium intakes had a less steep slope (response of serum 25(OH)D)

compared to those with higher mean calcium intakes (the two slopes were significantly different; 0.030 versus 0.044, respectively; *P*=0.023). Therefore, achievement of a serum 25(OH)D of 50 nmol/L required a lower vitamin D intake in those with a higher mean calcium intakes (≥1092 mg/d) than those with the lower mean intakes (<1092 mg/d). The significance of the interaction between total vitamin D intake and category of calcium intake was also tested in a regression model (*P*=0.03). The 95% lower Confidence Interval (CI) of the regression lines (as per the IOM approach) would predict total vitamin D intakes of 129 and 35 IU/d (equivalent to 3.2 and 0.9 μg/d, respectively) at the 30 nmol/L serum 25(OH)D threshold for the below and above than median calcium intake groups of RCT arms, respectively. However, the use of 95%CI versus 95% Prediction Intervals (PI) in the regression models allow prediction at the average (EAR) and at the 97.5th percentile (RNI), respectively (Cashman & Kiely, 2014). Thus, the 95% lower PI would be more appropriate for the estimation of a population reference intake, such as the RNI, but its generation from meta-regression data is only an approximation and it lends itself much more to generation from individual data from an RCT. As per our research strategy and approach, we awaited the outcome of the Part B *de novo* RCT to confirm an effect of calcium before we would try and generate a 95% lower PI stratified by calcium intake grouping.

Table 7 Study characteristics of randomized controlled trials carried out in the Winter months, located north of 46˚N/S included in meta-regression analysis.

Source (reference)*	Country	Latitude	Age range	% Male	Duration	Participants	Supplemental vitamin D_3 dose	Habitual vitamin D intake	Total vitamin D intake	Total Ca intake	Baseline 25(OH)D	Achieved 25(OH)D	Study period	Randomization	Dose checked	Compliance reported	Analytical method
		˚N /S	(y)		(wks)	*n*	(IU/d)	(IU/d)	(IU/d)	(mg/d) (inc supps given)	nmol/L	nmol/L	(Mos)	reported			
1	Finland	61	8-10 (9)[3]	45	56	24	400	200[5]	600	1092[5]	49.2 ± 19.0[4]	77.9 ± 23.7[4]	Jan-Mar	Y	N	N	CPBA
						27	0	200[5]	200	1092[5]	45.9 ± 15.5	43.2 ± 19.5					
2	Germany	52	70+ (74)	0	8	74	800	128[6]	928	2190[6]	25.7 ± 13.6	64.8 ± 27.4	Mar-May	Y	N	Y	RIA
						77	0	128[6]	128	2190[6]	24.6 ± 12.1	44.4 ± 27.4					
3	UK	55	18-27 (21)	50	8	15	600	65	665	2249	47.9 ± 16.0	85.6 ± 24.5	Jan-Mar	Y	N	N	EIA
						12	0	96	96	2383	55.5 ± 18.6	48.3 ± 16.8					
4	Finland	61	65-85 (71)	0	12	13	800	388	1188	1152	44.1 ± 13.5	70.2 ± 13.5	Jan-Apr	Y	N	N	HPLC
						11	400	424	824	911	46.5 ± 10.2	60.4 ± 10.2					
						13	200	388	588	1225	46.0 ± 10.2	55.0 ± 10.2					
						12	0	436	436	946	52.2 ± 19.9	43.9 ± 19.9					
5	Ireland	51	20-40 (30)	50	22	53	600	144	744	1014	75.9 ± 24.8	69.0 ± 18.6	Sept/Oct-Mar/Apr	Y	Y	Y	EIA
						57	400	140	540	976	72.2 ± 26.8	60.0 ± 13.4					
						48	200	172	372	905	60.0 ± 29.4	49.7 ± 11.4					
						57	0	136	136	924	65.7 ± 26.5	37.4 ± 12.2					
6	Ireland	51	64+ (71)	40	22	48	600	192	792	976	55.1 ± 22.8	73.8 ± 20.0	Sept/Oct-Mar/Apr	Y	Y	Y	EIA
						53	400	168	568	751	54.3 ± 21.8	69.5 ± 17.0					
						48	200	164	364	866	51.8 ± 22.1	53.2 ± 17.0					
						55	0	188	188	976	58.8 ± 25.9	41.6 ± 17.1					
7	Antartica	78	42	59	20	18	2000	302	2302		45.0 ± 14.0	71.0 ± 23.0	Mar-Aug	Y	Y	Y	RIA
						19	1000	329	1329		44.0 ± 19.0	63.0 ± 25.0					
						18	400	356	756		44.0 ± 18.0	57.0 ± 15.0					
						7	0	334	334		36.0 ± 17.0	34.0 ± 12.0					

8	Finland	61	21-49 (29)	100	25	16 16 16	800 400 0	34 304 264	1144 704 264	1510 1260 1250	60.3 ± 11.6 62.3 ± 13.6 64.7 ± 18.5	90. ± 11.6 75.6 ± 13.6 52.2 ± 18.5	Nov-Apr	Y	Y	Y	EIA
9	Finland & Denmark	58[8]	11.4	0	52	49 49 46	400 200 0	156 156 148	556 356 148	1197 1158 1052	58.4 ± 13.9 57.4 ± 12.4 54.5 ± 14.8	58.8 ± 10.9 47.6 ± 8.0 31.0 ± 10.0	Sept-Mar	Y	Y	Y	HPLC
10	Ireland	51	50+ (57)	45	10	13 16	800 0	304 260	1104 260	1114 970	49.7 ± 16.2 42.7 ± 12.6	69.0 ± 8.7 41.2 ± 11.1	Jan-Mar	Y	Y	Y	ELISA
11	New Zealand	46	18-50 (29)	21	25	24 25	1000 0	100[7] 100[7]	1100 100	869 869	80.0 ± 12.5 81.0 ± 21.7	80.0 ± 20.0 37.0 ± 16.6	Mar-Sept (NZ)	Y	Y	Y	Isotope dilution liquid chromatography + tandom MS

Abbv: years, y; months, mos; weeks, wks; days, d; international units, IU; January, Jan; March, Mar; April, Apr; September, Sept; October, Oct.

*List of references: 1) Ala-Houhala *et al*., 1988. 2) Pfeifer *et al.,* 2001. 3) Barnes *et al*., 2006. 4) Viljakainen *et al*., 2006 5) Cashman *et al*., 2008. 6) Cashman *et al*., 2009. 7) Smith *et al.,* 2009. 8) Viljakainen *et al*., 2009. 9) Cashman *et al.,* 2011. 10) Cashman *et al*., 2012. 11) Logan *et al*., 2012.

[1]Dose of vitamin D confirmed independently by analysis.

[2]Analytical method for circulating 25(OH)D: enzyme linked immune assay, EIA; radioimmunoassay, RIA; competitive binding protein assay, CBPA; high performance liquid chromatography, HPLC.

[3]Mean age is depicted in parentheses, and on its own in the case that an age range is not given.

[4]Mean ± standard deviation.

[5] Intake estimated from Andersen et al., 2006.

[6] Intake estimated from Flynn et al., 2009.

[7]Intake estimated from Nowson et al., 2012.

[8]An average latitude was taken from Helsinki, Finland (61N) and Copenhagen, Denmark (55°N).

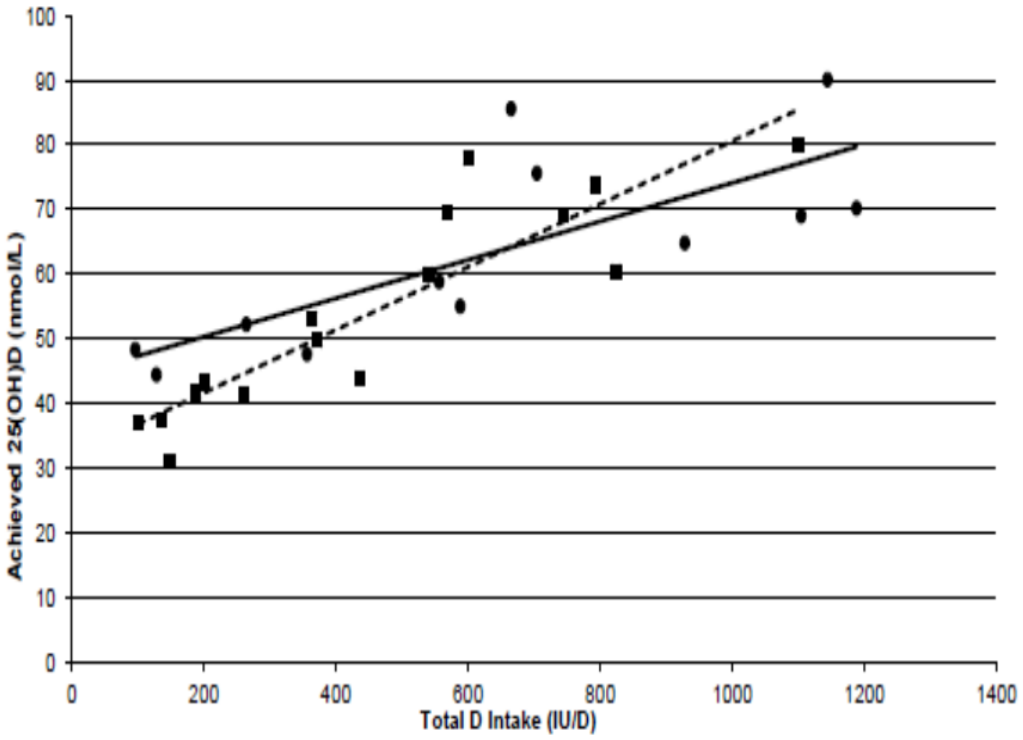


Figure 1. The relationship between total vitamin D intake and achieved serum 25(OH)D during winter stratified by category of calcium intake (dashed line representing data from RCTs with mean intakes below median [1092 mg/d]; full line representing data from RCTs with mean intakes above median.

3.2.4 Discussion of Part A findings

Overall, the *post hoc* analysis of data from the four individual vitamin D RCTs in Part A may suggest very little, if any, effect of habitual calcium intake on the response of serum 25(OH)D to vitamin D intake (and thus vitamin D requirements). This is caveated by the fact that the four vitamin D RCTs used in the present analysis were not designed to test the effect of calcium intake on dietary vitamin D requirements. The mean habitual calcium intake in the three population groups investigated were relatively good (mean/median 1122, 976 and 874 mg/d, for girls, young adults and older adults, respectively) and thus the numbers with calcium intakes below the lower cut-offs (550 and 700 mg/d), which are important public health-related intakes, were relatively low (*n* typically in range of 1 to ~20). This is an important limitation in terms of drawing conclusions on the impact of dietary calcium on response of serum 25(OH)D and thus dietary requirement estimates for vitamin D. Using data from all subjects within an age-group, regression analysis also suggested no interaction between habitual calcium intake on the response of serum 25(OH)D to vitamin D intake.

The interaction between vitamin D and dietary calcium was also tested using existing RCT data but from a wider selection of vitamin D RCTs in the meta-regression approach. The data used in the meta-regression was a mix of winter-based RCTs in children (*n* 2), adults (up to 50 y; *n* 4) and older adults (>50 y; *n* 5) and the analysis suggested an interaction between the response of winter serum 25(OH)D to total vitamin D and dietary calcium category (mean < and ≥ 1092 mg/d). Using a target serum 25(OH)D of 50 nmol/L (which the IOM suggest covers the needs of 97.5% of the population in terms of bone health), then those with a higher mean calcium intakes (≥1092 mg/d) required a lower vitamin D intake compared to those lower mean intakes (<1092 mg/d). It is important to stress that the cut-off was a median of group mean calcium intakes and thus reflected more a delineation of high calcium intake from lower intake. It is possible that the significant interaction in this meta-regression approach may be related to the relatively unadjusted analysis. In the analysis of our individual vitamin D RCTs some of the significant differences in response of serum 25(OH)D to different doses of vitamin D, especially in 64+ year olds became non-significant when adjusted for smoking, BMI, age and gender. Data on possible confounding variables were not sufficient in all the RCTs selected for the meta-regression such that they could to be included.

Overall, the data from Part A underscored the need for the hypothesis that calcium intake modifies vitamin D requirement be tested more vigorously in a RCT specifically designed

to address this question. This is Part B of the present work, and we indicated that should the hypothesis be confirmed by the new RCT, we would return to the individual data from our previous vitamin D RCTs and generate RNI estimates for those with moderate-low and high calcium intakes.

3.3 Part B -*De novo* Vitamin D × Calcium RCT

3.3.1 Specific objective

To conduct a randomised, placebo-controlled, single-dose vitamin D_3 RCT in apparently healthy, free-living adults (aged 50+ years) to investigate whether different levels of habitual calcium intake ranging from low-moderate to high (<EU EAR [550 mg/d] and >US RDA [1000 mg/d, dependent on age]), influence serum 25(OH)D concentrations, and also indices of vitamin D activation and catabolism as explanatory variables, during winter, when vitamin D intake is adequate versus inadequate.

3.3.2 Subjects and methods

3.3.2.1 Recruitment of study subjects

A total of 125 apparently healthy, free-living adults aged ≥50 years were recruited to this 15-week vitamin D_3 intervention trial. Subjects were recruited following a strong advertising campaign in the Cork catchment area, which included adverts on both radio and newspaper and also through the placement of posters on the campus of University College Cork and across the location. The recruitment phase began in September 2012. We aimed to recruit a ratio of approximately 2.5:1 women to men, as this was reflective of the ratio of women to men with a 'moderate-low' (<700 mg/d) habitual dietary calcium intake as reported in the National Adult Nutrition Survey in Ireland (IUNA, 2011) and also the National Diet and Nutrition Survey, UK (Henderson *et al*, 2003). Adults that were interested in participating in this study contacted a research nutritionist in the *Cork Centre for Vitamin D and Nutrition Research* who explained in detail the purpose of the study and what would be required of the participant for the duration of the trial should they decide to participate. Potential volunteers were also assessed for inclusion and exclusion criteria as follows:

White men and women aged ≥50 years that provided consent were included in the study. Those who were not willing to suspend the consumption of vitamin D supplements for the 4 weeks prior to the first visit and throughout the study period were excluded. Volunteers were also excluded if they planned to take a winter vacation to a location of either low latitude or high altitude (e.g., a winter sun or a mountain ski holiday) at which it would be

expected that there would be significant dermal production of vitamin D due to exposure to UVB radiation. Other exclusion criteria included a severe medical illness, hypercalcaemia, a known intestinal malabsorption syndrome, use of tanning facilities of any type, excessive alcohol use or the use of contraindicated medications known to alter the metabolism of vitamin D.

The study was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals, University College Cork. All participants gave their written consent according to the Helsinki Declaration. This trial was registered at clinicaltrials.gov as NCT01990872.

3.3.2.2 Design and conduct of study

This study was a double-blind, randomised, placebo-controlled vitamin D intervention trial. Subjects' habitual calcium and vitamin D intakes were assessed initially during the recruitment phase using a validated food frequency questionnaire that was administered by a research nutritionist. Subjects were stratified according to their habitual calcium intake, in this case a habitual calcium intake of <700 mg/d was considered to be 'moderate-low' and >1000 mg/d to be 'high' and subjects were then randomised to receive either placebo or 20 µg vitamin D_3 daily for 15 weeks throughout winter. Supplements were identical in appearance and taste. The contents of supplements used were confirmed using an in-house laboratory high performance liquid chromatography (HPLC) analysis at University College Cork.

The Reference Nutrient Intake and Population Reference Intake value for calcium is 700 mg/d in the UK and EU, respectively. According to the NDNS, approximately 49% men and 69% women (aged 50-64y) in the UK have calcium intakes below this reference intake level. The EAR for calcium in the EU is 550 mg/d, in the UK population there is approximately 5% men and 15% women with intakes below this intake level. In relation to the high calcium intake, North American IOM guidelines have suggested the EAR and RDA for calcium for women (>50 y) and men (>70 y) are 1000 mg/d and 1200 mg/d respectively. For men (aged 50-70 y), the RDA for calcium is 1000 mg/d (IOM, 2011). In addition for the purposes of informing the design of the present RCT, we used the data from Part A. Stratification of the RCTs by the median (1092 mg/d) of the reported average calcium intakes in these studies showed that there was a significant (P<0.05) interaction between calcium intake (above or below 1092 mg/d) and vitamin D intake such that those with lower calcium intakes had a less steep slope (response of serum 25(OH)D) compared to those with a higher calcium intake (the two slopes were significantly different; 0.030 versus 0.044, respectively; P=0.023). Therefore, achievement of a serum 25(OH)D of 50

nmol/L required a lower vitamin D intake in those with a higher calcium intakes (>1092 mg/d) than those with the lower intake (<1092 mg/d).

To maximise subject recruitment in the study, any subject (*n* 38) whose habitual calcium intake was above 700 mg/d but did not reach the >1000 mg/d target was asked during recruitment if they would take additional calcium. Subjects were given a choice as to whether they wished consume the additional calcium in the form of a supplement (one Calcichew tablet containing 500 mg calcium alone [Shire Pharmaceuticals Ireland Ltd, Citywest Business Campus, Dublin 24, Ireland]; *n* 36 opted for this route) or as additional milk in their diet (*n* 2 for this route), so that their calcium intake would be greater than 1000 mg/d for the duration of the intervention phase. This additional calcium intake was started four weeks before the intervention phase commenced to allow for normalisation of calcitropic hormone levels (Dawson-Hughes *et al*, 1993).

The IOM have suggested that the 'RDA-like' serum 25(OH)D concentration required to meet the needs of 97.5% of the population aged 1 year + is 50 nmol/L (IOM, 2011). On the basis of this serum concentration of 25(OH)D, the IOM has established the RDA for vitamin D at 15 and 20 μg vitamin D daily for those aged 1-70 years and >70 years, respectively, in North America (IOM, 2011). We have previously reported that a vitamin D RDA estimate of 24.7 μg/d was needed to maintain wintertime serum 25(OH)D over 50 nmol/L in 97.5% of adults aged 64+ y residing in Ireland (51-55^{o}N) (Cashman *et al*, 2009), and confirmed again recently in our RCT with vitamin D versus 25-hydroxyvitamin D in those aged 50+ years (Cashman *et al*, 2012). Therefore, we choose to use 20 μg/d supplemental vitamin D_3, which together with a habitual intake of vitamin D of ~5 μg/d in Irish adults (IUNA, 2011), would provide about 25 μg/d in the present study.

The randomisation of subjects to treatment groups was centralised and computer generated, and habitual calcium intake grouping (as per above) and sex were controlled for. The flow of subjects throughout this study can be seen in **Figure 2**.

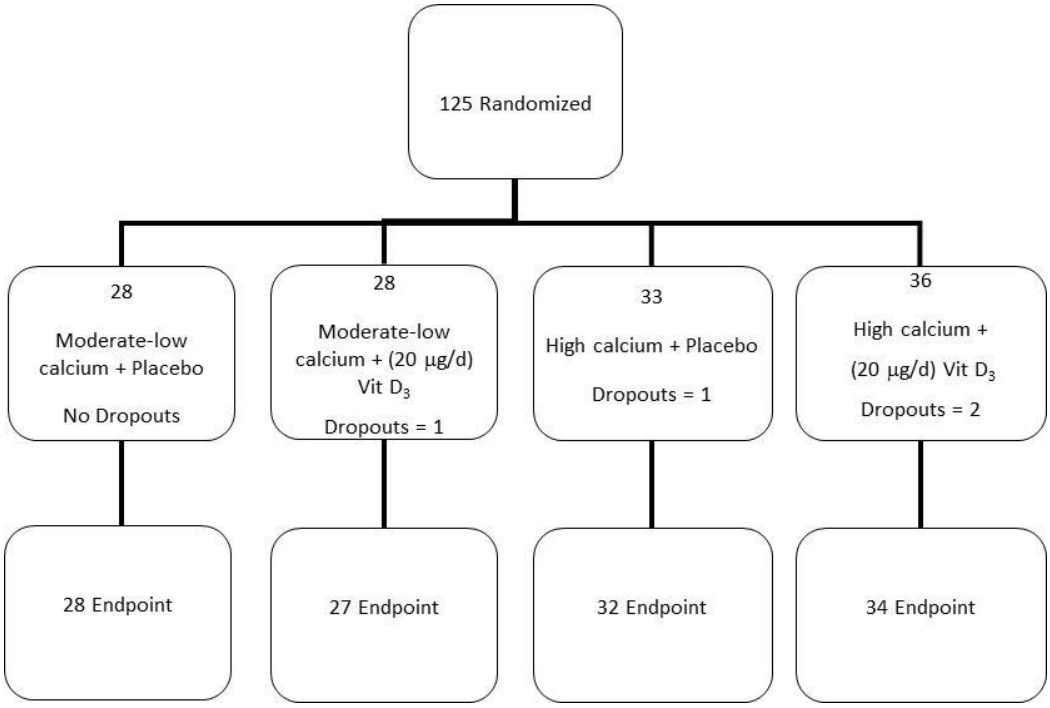


Figure 2. Flow of subjects through the study.

3.3.2.3 Laboratory analysis

Serum 25-hydroxyvitamin D.

The concentrations of total 25-hydroxyvitamin D [25(OH)D] (i.e., $25(OH)D_2$ plus $25(OH)D_3$) in all serum samples were measured by the *Vitamin D Research Group* at University College Cork using a liquid chromatography-tandem mass spectrometry (LC-tandem MS) method, described in detail elsewhere (Cashman *et al*, 2013). The quality and accuracy of serum 25(OH)D analysis by the LC-tandem MS in our laboratory is monitored on an ongoing basis by participation in the Vitamin D External Quality Assessment Scheme [DEQAS, Charing Cross Hospital, London, UK]. In addition, the *Vitamin D Research Group* is a participant in the Vitamin D Standardization Program (VDSP) (Sempos *et al*, 2012) and Vitamin D Standardization Certification Program (VDSCP) (Rahmani *et al*, 2013).

Serum intact parathyroid hormone.

Serum PTH concentrations were measured at University College Cork in all serum samples using an ELISA [Intact parathyroid hormone, MD Biosciences Inc., St. Paul, MN 55108]. The intra- and inter-assay CV was 3.4% and 3.8%, respectively.

Serum total calcium.

Total calcium and albumin concentrations in all serum samples were measured at Cork University Hospital, Cork, Ireland. Serum calcium concentrations were adjusted for albumin concentration.

Serum 1,25-dihydroxyvitamin D.

1,25-dihydroxyvitamin D [$1,25(OH)_2D$] concentrations were measured at University College Cork in baseline and endpoint serum samples using an ELISA [IDS 1,25-dihydroxyvitamin D EIA, Immuno Diagnostic Systems, Ltd., Boldon, UK]. The intra- and inter- assay CV for the ELISA method was 10.5% and 17.1%, respectively.

Serum 24,25-dihydroxyvitamin D.

The concentrations of $24,25(OH)_2D$ in baseline and endpoint serum samples were measured by the *Vitamin D Research Group* at University College Cork using an in-house LC-tandem MS method which was a modification of the method for measurement of 25(OH)D as described elsewhere (Cashman *et al*, 2013). The following summarized the modifications made to the original LC-tandem MS method for serum 25(OH)D. $24,25(OH)_2D_3$ and stable isotope labelled d6-$24,25(OH)_2D_3$ were purchased from Isosciences (4667 Somerton Road, Trevose, PA 19053, USA). In-house serums were used for low and high QC materials. The chromatographic column was a Supelco Ascentis Express F5 (10cm x 2.1mm, 2.7μm)

available from Sigma-Aldrich. Following addition of internal standard to the sample and adequate mixing, an aqueous solution of zinc sulphate was added to aid in the release of analytes from the vitamin D binding protein. Methanol was used to precipitate the proteins while a hexane/ethyl acetate (50:50) was the extraction solvent. Following extraction, samples were dried and reconstituted in UPLC mobile phase. Quality control serums were extracted and analysed in parallel to samples and were strategically placed close to the beginning, middle and end of the analysis on the LC-tandem MS instrument. Chromatographic resolution of 24,25$(OH)_2D_3$ was achieved on an Ascentis Express F5 column using an isocratic mixture of 32:68 A:B at 0.45 ml/min (where mobile phase A was 0.1% formic acid + 2 mM ammonium acetate in water and B was 0.1% formic acid + 2 mM ammonium acetate in methanol). A gradient with increased organic composition was applied towards the end of the 10 minute chromatographic run to wash out organic materials followed by a re-equilibration to initial conditions. MS detection was achieved on the Waters Acquity triple quadrupole detector through electrospray ionisation in positive ion multiple reaction monitoring (MRM) mode. Nitrogen was the desolvation gas and high purity Argon (BOC Ltd, Ireland) was the collision gas.

The ion MRM transition monitored for d6-24,25(OH)2D3 was m/z 417.3 → m/z 121.1. The quantifier transition for 24,25(OH)2D3 was m/z 417.3 → m/z 363.3 with m/z 417.3 → m/z 121 being used as a confirmatory transition. The inter-assay CV of the method was <5%, while the intra-assay CV was <12%.

Serum vitamin D binding protein.
Vitamin D Binding Protein (DBP) concentrations were measured at University College Cork in baseline and endpoint serum samples using an ELISA [Human Vitamin D BP Quantikine ELISA Kit, R&D Systems, USA]. The intra- and inter- assay CV for the ELISA method was 6.2% and 7.4%, respectively.

Estimation of serum free 25(OH)D concentrations and 24,25$(OH)_2$D:25(OH)D ratio
It has been suggested that free bioavailable 25(OH)D may provide for a more meaningful marker of vitamin D function (Chun *et al*, 2014). For example, an individual with inadequate serum total 25(OH)D (<50 nmol/L), might nevertheless have adequate levels of free 25(OH)D if serum levels of DBP are low (Chun *et al*, 2014). Therefore, we calculated the concentration of free 25(OH)D using a previously described equation (Bolland *et al*, 2007). The ratio of 24,25$(OH)_2$D to 25(OH)D has also been suggested as possible sensitive marker of vitamin D status and possess clinical utility as a marker for vitamin D catabolism because its production is reduced in the early stages of vitamin D deficiency and may be increased when vitamin D

supply is high, as the first step in the catabolic pathway of 25(OH)D (Schoenmakers *et al*., 2010; Wagner *et al*. 2011).

3.3.2.4 Statistical analysis

Berlin & Björkhem (1988) reported a mean difference in winter serum 25(OH)D of 17 nmol/L in Swiss men supplemented compared to un-supplemented with calcium. Stratification of vitamin D RCTs included in a previous meta-analysis by the *Cork Centre for Vitamin D and Nutrition Research* of functional markers of vitamin D status by whether vitamin D was administered alone or in conjunction with calcium (Seamans & Cashman, 2009) suggested a difference in average group mean serum 25(OH)D between groups receiving vitamin D alone compared with calcium plus vitamin D of ~13 nmol/L. Therefore, we used a more conservative 10 nmol/L difference as what we would need to be able to detect between calcium intake groups. On the basis of the distribution of winter time serum 25(OH)D data from older adults from a previous study by the *Cork Centre for Vitamin D and Nutrition Research* (Cashman *et al*. 2012), a study design recruiting 30 volunteers/group (which included 20% to cover potential drop-outs) will have 90% power to detect a 10 nmol/L difference in serum 25(OH)D between groups at α=0.5.

Statistical analysis of the data was conducted using SPSS© for Windows™ Version 22.0 (SPSS, Inc., Chicago, IL, USA). The distributions of all variables were tested with Kolmogorov-Smirnov tests. Descriptive statistics (means ± SD or medians and interquartile range [IQR], when appropriate) were determined for all variables. Dietary vitamin D, serum PTH, 24,25$(OH)_2$D, free 25(OH)D, and 1,25$(OH)_2$D were not normally distributed and thus were either log or square root transformed to achieve near-normal distributions. Serum concentrations of 25(OH)D, albumin-corrected calcium, DBP, 24,25$(OH)_2$D:25(OH)D ratio as well as age, weight, height, BMI and dietary calcium were normally distributed. Baseline characteristics of male and female subjects were compared using unpaired Student's *t* tests. Baseline characteristics of subjects in the different intervention groups were compared using Chi-square (for ratio of men to women) and one-factor analysis of variance (ANOVA). Linear models of the response in a repeated measures analysis for the differences in serum 25(OH)D, albumin-corrected calcium, PTH, 24,25$(OH)_2$D, 1,25$(OH)_2$D, 24,25$(OH)_2$D:25(OH)D, free 25(OH)D, and DBP concentrations were constructed. The linear models included three- and two-way interactions between the main effects (calcium intake grouping and vitamin D treatment) and these were reported. If no significant calcium intake grouping × vitamin D treatment × time interaction was observed, then calcium intake grouping × time and vitamin D treatment × time interactions were explored. The models were also run to include sex as an

additional factor. Bonferroni-adjusted *t* tests were used for *post hoc* analysis to the ANOVA. A *P*-value of <0.05 was taken as being statistically significant.

3.3.3. Results of Part B

3.3.3.1 Baseline characteristics of subjects in de novo RCT

Of 125 subjects recruited onto the study, 121 subjects completed the intervention. The progress of these subjects through the trial is shown in Figure 2. The baseline characteristics of those subjects who entered the intervention are shown in **Table 8**. While women were, on average, lighter and smaller than men (both $P<0.0001$) and had lower serum $1,25(OH)_2D$ concentrations ($P<0.05$), there was no difference ($P>0.1$) in mean age, BMI, habitual intakes of vitamin D and calcium, serum 25(OH)D, albumin-corrected calcium, PTH, $24,25(OH)_2D$, DBP, or free 25(OH)D concentrations between men and women (data not shown).

3.3.3.2 Effects of intervention with vitamin D_3 in moderate-low- and high- calcium intake groups

There was no difference ($P>0.3$) in mean age, weight, height, or BMI at baseline among the four treatment groups (placebo and vitamin D_3 with moderate-low calcium intake groups; placebo and vitamin D_3 with high calcium intake groups) (data not shown). Similarly, there was no significant difference in the proportion of men to women among the treatment groups (**Table 9**). Mean habitual dietary vitamin D was significantly ($P=0.01$) higher in the group with 'high calcium' intake who were randomised to receive vitamin D supplementation compared to the 'moderate-low' calcium intake group who were randomised to receive placebo, but otherwise there was no significant difference (Table 9). Mean habitual dietary calcium was similar among the two 'moderate-low' calcium intake groups and similar among the two 'high calcium' intake groups, with the latter two groups significantly higher calcium intake ($P<0.0001$) than the former two groups (Table 9). The mean total calcium intakes (habitual intake plus additional calcium intakes) for the two 'high calcium' intake groups are shown in Table 9, and these were similar ($P>0.7$).

There were no adverse events reported during the study. Of the 4 dropouts, two subjects were in the period between baseline and midpoint sampling (one subject was from the 'moderate-low' calcium intake plus 20 μg vitamin D_3/d group, one from the 'high calcium' intake plus placebo group), and two subjects (both from the 'high calcium' intake plus 20 μg vitamin D_3/d group) were in the period between midpoint and endpoint sampling. Dropout during the intervention phase was for reasons of unavailability or loss of interest, and in no instance was dropout related to the intervention.

Table 8. Baseline characteristics of the subjects who entered the intervention study[1].

n	125
Male:Female	35:90
Age (y)	59.9 ± 6.5
Weight (kg)	72.5 ± 13.3
Height (m)	1.64 ± 0.11
BMI (kg/m^2)	26.7 ± 4.2
Dietary calcium (mg/d)	814 ± 413
Dietary vitamin D (µg/d)	4.4 [2.9, 6.8]
Serum 25(OH)D (nmol/L)	55.1 ± 20.4
Serum calcium (mmol/L)[2]	2.3 ± 0.1
Serum PTH (pg/mL)	46.1 [35.9, 60.3]
Serum 1,25$(OH)_2$D (pmol/L)	77.6 [56.9, 99.6]
Serum 24,25$(OH)_2$D (nmol/L)	2.23 [1.24, 3.39]
Serum DBP (mg/L)	216 ± 74
Serum free 25(OH)D (pmol/L)	17.3 [13.2, 24.7]

DBP, vitamin D binding protein.

[1]Values represent means ± SD or medians [interquartile range] in the case of non-normally distributed parameters.

[2]Albumin corrected.

Table 9. Dietary intakes of vitamin D and calcium and serum 25(OH)D; calcium and, PTH concentrations among treatment groups at baseline, mid-point and end-point of the 15 week-intervention in apparently healthy older adults[1]

Calcium intake group	Low Calcium (<700 mg/d)		High Calcium (>1000 mg/d)		Repeated measures ANOVA		
Vitamin D_3 treatment groups	Placebo	20 µg Vitamin D_3/d	Placebo	20 µg Vitamin D_3/d	*P*[5]	*P*[6]	*P*[7]
n (at endpoint)	28	27	32	34			
Sex (male:female)[2]	8:20	8:19	8:24	10:24			
Habitual dietary vitamin D (µg/d)[2,3]	3.0 [2.2, 5.5][a]	4.0 [2.8, 6.6][ab]	3.8 [3.2, 6.1][ab]	6.1 [4.3, 9.2][b]	-	-	-
Dietary calcium (mg/d) [2,4]:							
Habitual	514 ± 156[a]	479 ± 169[a]	1061 ± 373[b]	1072 ± 389[b]	-	-	-
Habitual + supplemental	514 ± 156[a]	479 ± 169[a]	1358 ± 254[b]	1337 ± 258[b]	-	-	-
Serum 25(OH)D (nmol/L):							
pre-intervention[8,9,10]	58.3 ± 16.7	54.3 ± 25.1	54.3 ± 16.7	54.3 ± 22.3			
mid-intervention	44.3 ± 15.8	76.1 ± 16.4	43.4 ± 16.0	73.0 ± 14.9			
post-intervention	42.1 ± 14.8	80.4 ± 18.7	41.4 ± 16.3	74.3 ± 15.4	0.202	0.840	<0.0001
Serum calcium (mmol/L)[11]:							
pre-intervention[8,9]	2.3 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	2.3 ± 0.1			
mid-intervention	2.4 ± 0.1	2.4 ± 0.1	2.3 ± 0.1	2.4 ± 0.1			
post-intervention	2.3 ± 0.1	2.4 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	0.494	0.971	0.310
Serum PTH (ng/mL):							
pre-intervention[8,9,12]	47.0 [35.6, 59.9]	53.7 [38.2, 70.5]	43.1 [35.0, 52.2]	42.8 [33.3, 59.9]			
mid-intervention	45.0 [40.5, 65.2]	52.9 [41.4, 64.4]	45.2 [39.2, 55.6]	43.0 [32.1, 54.3]			
post-intervention	50.2 [42.2, 61.8]	47.0 [37.7, 61.4]	46.5 [39.4, 55.9]	38.3 [32.1, 57.1]	0.306	0.726	0.013

Table 9 continued…

[1]25(OH)D, 25-hydroxyvitamin D; PTH, parathyroid hormone.
[2]Chi-square test was used to test differences for between-group differences in case of sex ($P>0.9$) and ANOVA was used in the case of dietary calcium (both $P<0.0001$) and vitamin D ($P<0.01$). [a,b]Different superscript letters represent significant differences among group means: $P<0.05$ (Bonferroni-adjusted *t* test).
[3]Medians [interquartile range] of non-normally distributed variable (all such values)
[4]Mean ± SD (all such values)
[5]Repeated-measures ANOVA was used to test the time × vitamin D treatment × calcium intake grouping interaction effect.
[6]Repeated-measures ANOVA was used to test the time × calcium intake grouping interaction effect.
[7]Repeated-measures ANOVA was used to test the time × vitamin D treatment interaction effect.
[8]There were no significant differences in baseline concentrations across the four groups ($P>0.4$ in all cases).
[9]All baseline blood samples taken between 5th and 23th November 2012 and all endpoint blood samples taken between 18th February and 14th March 2013.
[10]Mean (± SD) serum 25(OH)D concentrations in the combined 20 µg Vitamin D_3/d groups (*n* 64), irrespective of calcium intake grouping, increased significantly from baseline by week 8 ($P<0.0001$; via Bonferroni-adjusted *t* test) and further by week 15 ($P=0.005$) (54.3 ± 23.4 to 74.3 ± 15.5 to 76.9 ± 17.1 nmol/L, respectively). Conversely, mean (± SD) serum 25(OH)D concentrations in the combined Placebo groups (*n* 61), decreased significantly from baseline by week 8 ($P<0.0001$) and further by week 15 ($P=0.002$) (56.1 ± 16.7 to 43.8 ± 15.8 to 41.7 ± 15.5 nmol/L, respectively).
[11]Albumin corrected.
[12]Median [interquartile range] baseline serum PTH in the two 'moderate-low calcium' intake groups combined (*n* 56) was significantly higher than that in the two 'high calcium' intake groups combined (*n* 69) (50.2 [38.2, 67.6] versus 43.9 [34.7, 55.9] pg/mL; $P=0.037$ via unpaired *t* test)

There was good supplement adherence on the basis of the pill count (median [IQR] compliance was 98.6% [94.3; 100%], and compliance was similar among the 4 treatment groups; *P*=0.9).

The effect of vitamin D treatment in the two calcium intake groupings ('moderate-low' versus 'high') on serum 25(OH)D, albumin-corrected calcium and PTH at baseline, midpoint and endpoint is shown in Table 9. There was no significant difference in pre-intervention serum 25(OH)D concentrations among the treatment groups (Table 9). Repeated measures ANOVA showed that there was no significant (*P*=0.2) time × vitamin D treatment × calcium intake grouping interaction effect on mean serum 25(OH)D concentration over the 15-wk intervention period (Table 9). Likewise, there was no significant (*P*=0.8) time × calcium intake grouping interaction effect, but there was a significant (*P*≤ 0.0001) time × vitamin D treatment interaction effect on mean serum 25(OH)D concentration over the 15-wk intervention period (Table 9). As calcium intake grouping had no effect, the mean (± SD) baseline serum 25(OH)D of the two groups combined who received 20 µg/d of vitamin D_3 (54.3 ± 23.4 nmol/L; *n* 64) was similar (*P*>0.6) to that of the two groups combined who received placebo (56.1 ± 16.7 nmol/L; *n* 61). Mean (± SD) serum 25(OH)D concentrations in the combined groups who received 20 µg/d of vitamin D_3, irrespective of calcium intake grouping, increased significantly (to 74.3 ± 15.5 nmol/L; *P*<0.0001) by approximately midpoint (week 8), and there was a modest, but significant (*P*=0.005), further increase (to 76.9 ± 17.1 nmol/L) by week 15. Conversely, mean (± SD) serum 25(OH)D concentrations in the combined groups who received placebo, irrespective of calcium intake grouping, decreased significantly (to 43.8 ± 15.8 nmol/L; *P*<0.0001) by week 8, and there was a modest, but significant (*P*=0.002), further decrease (to 41.7 ± 15.5 nmol/L) by week 15.

There was no significant interaction between time × vitamin D treatment × calcium intake grouping (or time × vitamin D treatment or time × calcium intake grouping) and sex (all *P*>0.4) in the response of serum 25(OH)D to intervention (data not shown).

There was no significant difference in pre-intervention serum albumin-corrected calcium concentrations among the treatment groups (Table 9). There was no significant time × vitamin D treatment × calcium intake grouping interaction effect or time × vitamin D treatment or time × calcium intake grouping interaction effects (all *P*>0.3) on mean serum albumin-corrected calcium concentrations over the 15-wk intervention period.

Repeated measures ANOVA showed that there was a significant (P<0.05) difference in pre-intervention serum PTH concentrations among the groups stratified by calcium intake. The two 'moderate-low calcium' groups combined (n 56) had a modest, but significantly (P=0.037), higher median (IQR) baseline serum PTH compared to that of the two groups combined (n 69) who had a 'high calcium' intake (50.2 [38.2, 67.6] versus 43.9 [34.7, 55.9] pg/mL, respectively). There was no significant (P=0.3) time × vitamin D treatment × calcium intake grouping interaction effect on median (IQR) serum PTH concentration over the 15-wk intervention period (Table 9). Likewise, there was no significant (P=0.7) time × calcium intake grouping interaction effect, but there was a significant (P=0.013) time × vitamin D treatment interaction effect on median (IQR) serum PTH concentration over the 15-wk intervention period (Table 9). Median serum PTH concentrations in the two groups who received 20 µg/d of vitamin D_3, irrespective of calcium intake grouping, remained unchanged (P>0.3) over the 15 weeks, whereas median serum PTH concentrations in the two groups who received placebo, irrespective of calcium intake grouping, remained unchanged (P>0.7) over the first 8 weeks but had increased significantly (P=0.034) by week 15 (44.8 [35.2, 56.9], 45.1 [40.1, 58.4], and 47.8 [49.0, 57.1] pg/mL, respectively).

The effect of vitamin D treatment in the two calcium intake groupings ('moderate-low' versus 'high') on serum 24,25$(OH)_2$D, ratio of 24,25$(OH)_2$D:25(OH)D, 1,25$(OH)_2$D, free 25(OH)D or DBP concentrations at baseline and endpoint is shown in **Table 10**. There was no significant difference in pre-intervention concentrations for any of these variables among the treatment groups (Table 10). Repeated measures ANOVA showed that there was no significant (P>0.1 for all) time × vitamin D treatment × calcium intake grouping interaction effect on mean serum 24,25$(OH)_2$D, ratio of 24,25$(OH)_2$D:25(OH)D, 1,25$(OH)_2$D, free 25(OH)D, or DBP concentration over the 15-wk intervention period (Table 10). Likewise, there was no significant (P>0.1 for all) time × calcium intake grouping interaction effect on mean serum 24,25$(OH)_2$D, ratio of 24,25$(OH)_2$D:25(OH)D, 1,25$(OH)_2$D, free 25(OH)D and DBP concentration over the 15-wk intervention period (Table 10). While there was no significant (P=0.7) time × vitamin D treatment interaction effect on mean serum DBP, there were significant ($P \leq 0.0001$ for all) time × vitamin D treatment interaction effects on mean serum 24,25$(OH)_2$D, ratio of 24,25$(OH)_2$D:25(OH)D, 1,25$(OH)_2$D, and free 25(OH)D concentrations over the 15-wk intervention period (Table 10).

As calcium intake grouping had no effect, the mean baseline 24,25$(OH)_2$D, ratio of 24,25$(OH)_2$D:25(OH)D, 1,25$(OH)_2$D, and free 25(OH)D of the two groups combined who received 20 µg/d of vitamin D_3 (*n* 61) was similar (*P*>0.2 for all) to that of the two groups combined who received placebo (*n* 60) (data not shown). The two placebo groups combined (*n* 60) had a significantly (*P*<0.0001) lower median (IQR) serum 24,25$(OH)_2$D at endpoint compared to that of the two vitamin D_3-supplemented groups combined (*n* 61) (1.14 [0.59, 1.85] versus 3.54 [2.87, 4.54] nmol/L, respectively). The two placebo groups combined (*n* 60) had a significantly (*P*≤0.0001) lower mean (± SD) serum 24,25$(OH)_2$D:25(OH)D ratio at endpoint compared to that of the two vitamin D_3-supplemented groups combined (*n* 61) (0.030 ± 0.014 versus 0.048 ± 0.014, respectively). The two placebo groups combined (*n* 60) had a significantly (*P*≤0.0001) lower median (IQR) serum 1,25$(OH)_2$D at endpoint compared to that of the two vitamin D_3-supplemented groups combined (*n* 61) (65.0 [48.2, 91.4] versus 99.7 [65.7, 119.1] pmol/L, respectively). The two placebo groups combined (*n* 60) had a significantly (*P*≤0.0001) lower median (IQR) serum free 25(OH)D at endpoint compared to that of the two vitamin D_3-supplemented groups combined (*n* 61) (2.93 [2.16, 4.65] versus 6.42 [4.77, 9.40] pmol/L, respectively). In terms of the within combined treatment group changes from baseline to endpoint, mean/median serum 24,25$(OH)_2$D, ratio of 24,25$(OH)_2$D:25(OH)D, and free 25(OH)D concentrations in the combined groups who received placebo (*n* 60), irrespective of calcium intake grouping, decreased significantly from week 0 to 15 (by 54.5, 28.6, and 24.3%, respectively; *P*≤0.0001 for all), whereas there was no significant change (*P*=0.4) in median serum 1,25$(OH)_2$D concentration over the 15 weeks. Mean/median serum 24,25$(OH)_2$D, ratio of 24,25$(OH)_2$D:25(OH)D, 1,25$(OH)_2$D, and free 25(OH)D concentrations in the combined groups who received 20 µg/d of vitamin D_3 (*n* 61), irrespective of calcium intake grouping, increased significantly from week 0 to 15 (by 85.6, 14.1, 24.1 and 61.8%, respectively; *P*<0.0007 for all).

Table 10. Serum 24,25$(OH)_2$D, 1,25$(OH)_2$D, free 25(OH)D and vitamin D binding protein concentrations and serum 24,25$(OH)_2$D: 25(OH)D ratio among treatment groups at baseline and end-point of the 15 week-intervention in apparently healthy older adults[1].

Calcium intake group	Low Calcium (<700 mg/d)		High Calcium (>1000 mg/d)		Repeated measures ANOVA		
Vitamin D_3 treatment groups	Placebo	20 µg Vitamin D_3/d	Placebo	20 µg Vitamin D_3/d	*P*[4]	*P*[5]	*P*[6]
n (at endpoint)	28	27	32	34			
Serum 24,25$(OH)_2$D (nmol/L)[2]:							
pre-intervention[7,8]	2.3 [1.8, 3.7]	1.5 [1.2, 4.0]	2.4 [1.3, 3.3]	1.9 [0.9, 2.8]			
post-intervention	1.3 [0.7, 1.9]	3.5 [2.7, 4.9]	1.0 [0.5, 1.7]	3.5 [2.9, 4.4]	0.794	0.769	<0.0001
Serum 24,25$(OH)_2$D:25(OH)D ratio[3]:							
pre-intervention[7,8]	0.041 ± 0.017	0.041 ± 0.021	0.043 ± 0.017	0.042 ± 0.020			
post-intervention	0.031 ± 0.014	0.047 ± 0.013	0.030 ± 0.014	0.049 ± 0.014	0.532	0.739	<0.0001
Serum 1,25$(OH)_2$D (pmol/L):							
pre-intervention[7,8]	79.9 [64.4, 103.6]	82.0 [60.5, 108.8]	59.5 [48.8, 82.8]	79.6 [57.0, 93.5]			
post-intervention	69.2 [52.1, 91.5]	103.9 [70.4, 122.6]	64.2 [46.4, 91.4]	96.2 [61.7, 109.8]	0.143	0.109	<0.0001
Serum free 25(OH)D (pmol/L):							
pre-intervention[7,8]	20.3 [15.6, 26.0]	17.5 [11.3, 28.1]	16.2 [11.5, 23.1]	17.3 [13.1, 25.65]			
post-intervention	15.1 [10.9, 20.5]	30.6 [22.1, 42.6]	11.5 [8.2, 18.0]	25.1 [19.7, 34.1]	0.160	0.819	<0.0001
Serum DBP (mg/L):							
pre-intervention[7,8]	211.4 ± 62.5	198.5 ± 77.2	242.5 ± 75.1	211.3 ± 75.8			
post-intervention	206.9 ± 58.9	195.2 ± 89.7	234.9 ± 80.3	208.2 ± 71.9	0.764	0.787	0.583

Table 10 continued…

[1]24,25$(OH)_2$D, 24,25-dihydroxyvitamin D; 1,25$(OH)_2$D, 1,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; VBP, vitamin D binding protein
[2]Medians [interquartile range] of non-normally distributed variable (all such values)
[3]Mean ± SD (all such values)
[4]Repeated-measures ANOVA was used to test the time × vitamin D treatment × calcium intake grouping interaction effect.
[5]Repeated-measures ANOVA was used to test the time × calcium intake grouping interaction effect.
[6]Repeated-measures ANOVA was used to test the time × vitamin D treatment interaction effect.
[7]There were no significant differences in pre-intervention concentrations across the four groups (P>0.1 in all cases).
[8]All baseline blood samples taken between 5th and 23th November 2012 and all endpoint blood samples taken between 18th February and 14th March 2013.
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3.3.3.2 Effects of intervention with vitamin D_3 in 'low' calcium versus 'high' calcium intake groups; secondary analysis of serum 25(OH)D

A secondary analysis of the data in which subjects with 'low' calcium intakes (i.e., cut-off set at <550 mg/d [mean ± SD, 391 ± 136 mg/d]; *n* 32) were compared with those with 'high' calcium intake (i.e., cut-off also set at >1000 mg/d but which was achieved from habitual intake alone and without any additional calcium needed in study period [mean ± SD, 1376 ± 371 mg/d]; *n* 31) showed that there was no significant (P=0.2) time × vitamin D treatment × calcium intake grouping interaction effect on mean serum 25(OH)D concentration over the 15-wk intervention period.

3.3.3.3 Effects of intervention with vitamin D_3 in 'moderate-low' calcium and 'high' calcium intake groups stratified by adequacy of baseline serum 25(OH)D concentration; secondary analysis of serum 25(OH)D

Further secondary analysis of the data was performed to see whether adequacy of baseline serum 25(OH)D concentrations influenced the main outcome. All subjects were stratified on the basis of baseline serum 25(OH)D concentrations (< and >40 nmol/L, a cut-off suggested by the IOM as the estimated average requirement (EAR)-like concentration). Mean (± SD) baseline serum 25(OH)D of the resulting subgroups was 31.1 ± 4.6 nmol/L (*n* 30) and 62.8 ± 17.2 nmol/L (*n* 92) for < and >40 nmol/L, respectively. Repeated measures ANOVA showed that there was no significant (P>0.2 for both) time × vitamin D treatment × calcium intake grouping interaction effect on mean serum 25(OH)D concentration over the 15-week intervention period in either subgroup (data not shown).

3.3.4 Discussion of Part B findings

Recent dietary requirement estimates for vitamin D from both sides of the Atlantic have prioritized winter-time as a critical period during which intakes of vitamin D should maintain serum 25(OH)D concentrations above chosen cut-offs (IOM, 2011; German Nutrition Society, 2012; Nordic Nutrition Recommendations, 2012). The IOM while setting their most recent DRI for vitamin D highlighted uncertainty and gaps in the available data about the influence of calcium intake on the regulation of vitamin D activation and catabolism (IOM, 2011). Thus, while as per convention the vitamin D DRI was established by the IOM on the assumption that the requirement for dietary calcium is being met (IOM, 2011), inadequacy in calcium intake could cause changes in the efficient handling of, or physiological response to, vitamin D that might not otherwise be present. A significant portion of adult populations in Europe and North America fail to meet respective dietary calcium requirements (Flynn *et al*, 2009; IOM, 2011; IUNA, 2011). In fact, the 2010 Dietary Guidelines Advisory Committee identified four nutrients of public health concern with calcium being one of them. Thus, clarifying whether low intakes of calcium increase the dietary requirement for vitamin D is important from a public health perspective and also in devising preventative strategies for vitamin D deficiency. Using a 15 week winter-based vitamin D_3 intervention study in 125 apparently healthy, free-living, white adults aged ≥50 years at a latitude of 51^{o}N, it was possible to examine the potential interactions of dietary calcium intake on both the decline of serum 25(OH)D over winter whilst on a habitual inadequate vitamin D intake as well as on the response of serum 25(OH)D to an intake aimed at achieving at least the IOM suggested 'RDA-like' 25(OH)D concentration of 50 nmol/L (IOM, 2011). It was found that responses in serum 25(OH)D (bound and free) concentrations throughout winter, as well as indices of vitamin D activation and catabolism as potential explanatory variables, were similar in older adults irrespective of whether they were on relatively low or high habitual calcium intakes.

These data would suggest that recently proposed dietary requirement estimates for vitamin D (IOM, 2011; German Nutrition Society, 2012; Nordic Nutrition Recommendations, 2012; Weggemans *et al.*, 2013; UK Scientific Advisory Committee on Nutrition [SACN] 2016) will ensure the adequacy of serum 25(OH)D concentrations in older adults even when the calcium intakes of these adults are in the inadequate range (<700 mg/d, and possibly even <550 mg/d). The winter-based, 2 × 2 factorial design of the present vitamin D_3 intervention study differs from those of the limited number of previous RCTs in this area (Berlin & Björkhem, 1988; Goussous *et al*, 2005; McCullough *et al*, 2009). It allowed

for the examination of the possible interaction effect of two different nutritionally and public health relevant calcium intake bands (i.e., <550/<700 versus >1000 mg/d) on the natural decline in serum 25(OH)D concentrations due to absent or markedly diminished UVB sunlight as well as on the potential of an adequate vitamin D intake in maintaining serum 25(OH)D concentrations ≥ 50 nmol/L (Holick *et al.*, 1973) during winter. Berlin & Björkhem (1988) reported that supplementation of healthy young adult men (*n* 14/group) with 2000 mg of calcium per day in addition to the usual diet for 6- to 7-weeks during late autumn/early winter in Sweden led to a significant increase in serum 25(OH)D concentrations (by 30%) relative to that in a control group on a normal diet. Unfortunately, the habitual dietary intake of calcium was not assessed in the study but the authors worked on the assumption that it was about 800 mg/d (Berlin & Björkhem, 1988). While none of the subjects received vitamin D supplementation, serum 25(OH)D concentrations increased in both the calcium and control group over the 6- to 7-week study period (increasing from a mean of 67 to 71 nmol/L in the control group) which was from late October to early December. Of note, the authors suggest that unpublished data of theirs on an identical trial, but one conducted from February to March, still suggested an elevatory effect of additional calcium on serum 25(OH)D (mean decrease in serum 25(OH)D of 21% versus 9% in control and calcium-supplemented groups, respectively $P<0.002$ [actual data not provided]. McCullough *et al*, (2009) also performed a randomized 2 × 2 factorial design pilot intervention study with vitamin D and/or calcium supplements in US-based (42°N) previous adenomatous colonic polyp patients (aged 30-74 y; 70% were men), as part of a chemoprevention trial of biomarkers of risk of colorectal adenoma. Four groups of patients, with group mean habitual calcium intakes in the range of 625-753 mg/d, were randomized to receive daily either placebo, an additional 2000 mg calcium, an additional 20 μg vitamin D_3, or an additional 2000 mg calcium plus 20 μg vitamin D_3 for 6 months (*n* 22/23 per group) (McCullough *et al*, 2009). The additional calcium did not influence the response of serum 25(OH)D to vitamin D supplementation. Baseline blood sampling occurred throughout the year, with only 26% during winter. Consequently, there was no significant reduction in mean serum 25(OH)D concentrations over the intervention period in the two groups not randomized to receive vitamin D supplements, whereas there was the expected significant increases in mean serum 25(OH)D in the two vitamin D-supplemented groups. Goussous *et al*, (2005) performed a RCT in which healthy older men and women (aged 50+ years; 74% female on average; mean habitual calcium, 577 mg/d) were randomized to receive calcium supplements (1000 mg/d) or placebo (*n* 23/29 per group) for 90 days throughout winter. However, as all participants received vitamin D_3 (20 μg/d) over the intervention period, neither group had a decline in mean winter serum 25(OH)D concentrations but instead had the expected

increase. The response of serum 25(OH)D to vitamin D_3 supplementation did not differ between the calcium or placebo group (Goussous *et al*, 2005).

The IOM used data from winter-based RCTs with vitamin D alone to establish the DRI for vitamin D (IOM, 2011). This is because of the concern that altering calcium intake as well as vitamin D intake in RCTs may impact on serum 25(OH)D concentration due to altered regulation of vitamin D activation and catabolism. All of the calcium-supplemented subjects in the three RCTs above (Berlin & Björkhem, 1988; Goussous *et al.*, 2005; McCullough *et al*, 2009) had increases in their usual calcium intake of the order of an additional 1000 to 2000 mg of calcium per day, likely bringing their total mean calcium intakes into the range of >1500 to >2700 mg/d. This perturbation in calcium intake alone may have altered calcitropic hormone levels from baseline to end-point. One study found a significant reduction in serum $1,25(OH)_2D$ (but not PTH) upon calcium supplementation (Berlin & Bjorkhem, 1988), while $1,25(OH)_2D$ (Goussous *et al*, 2005; McCullough *et al*, 2009) and surprisingly PTH (Goussous *et al*, 2005) were unaffected by calcium supplementation in the other studies. The response of these hormones to intervention are variable (Goussous *et al*, 2005). In the present study, we tried to minimize the perturbation of usual calcium intake and possible knock-on effects on calcitropic hormones, by stratifying at recruitment by habitual calcium intake and then only in those (*n* 38) falling short of the high calcium intake cut-off (>1000 mg/d) did we add supplemental calcium to meet this target (started four weeks prior to baseline to facilitate normalisation of calcitropic hormone levels (Dawson-Hughes *et al*, 1993). In the comparison of subjects with <550 mg/d and >1000 mg/d, there was no perturbation as these were all habitual intakes of calcium. Thus, the declines and increases in serum 25(OH)D over winter which were associated with inadequate and adequate dietary vitamin D, respectively, were compared in subjects on their habitual low calcium intakes and on their habitual, or as close as, high calcium intakes.

Using $24,25(OH)_2D$ concentration and the ratio of $24,25(OH)_2D$:25(OH)D as markers for vitamin D catabolism (Schoenmakers *et al*, 2010; Wagner *et al*, 2011; Jones, 2013), the lack of effect of level of habitual intake of calcium on response of both during the present study is in keeping with the main findings of a lack of interaction between calcium and vitamin D on response of serum 25(OH)D. In contrast, supplementation with vitamin D_3 significantly increased serum $24,25(OH)_2D$ concentration and the ratio of $24,25(OH)_2D$:25(OH)D, as well as serum 25(OH)D, suggesting induction of the catabolic pathway via increased 24-hydroxylase activity. Serum $1,25(OH)_2D$ has been suggested as a key regulator of catabolism of 25(OH)D (Clements *et al*, 1987; Clements *et al*, 1992;)

whereas it appears less clear whether or not PTH itself has an independent effect on catabolism of 25(OH)D (Clements *et al*, 1987). Vitamin D_3 supplementation during winter had no effect on serum PTH in the present study, but significantly increased serum $1,25(OH)_2D$ concentrations. While serum $1,25(OH)_2D$ was not increased by vitamin D supplementation in some RCT (Goussous *et al*, 2005; McCullough *et al*, 2009), it has in others (Harris & Dawson-Hughes, 2002). Interestingly, serum $24,25(OH)_2D$ concentration and the ratio of $24,25(OH)_2D$:25(OH)D were significantly reduced during winter in the placebo groups, suggesting a possible a 24-hydroxylase mediated sparing effect aimed at maintaining serum 25(OH)D; even though concentrations, as expected, did decrease during winter. While serum PTH was significantly increased over winter-time in the placebo groups, serum $1,25(OH)_2D$ was unchanged.

While the present study did not include an entire group of subjects with extremely low calcium intakes where perhaps an effect on serum 25(OH)D catabolism may occur, and thus may be a potential limitation, 50% of subjects in the low-moderate calcium intake grouping had intakes <550 mg/d and 25% had intakes <400 mg/d, and all had <700 mg/d. At a population level, only 3% and 13% of Irish adults have calcium intakes <400 and <550 mg/d, respectively (IUNA, 2011).

In conclusion, the findings of the Part B of the present work suggest that responses in serum 25(OH)D (both bound and free) concentrations throughout winter, as well as indices of vitamin D activation and catabolism, were similar in older adults irrespective of whether they were on relatively low or high habitual calcium intakes. Thus, recent dietary vitamin D requirement estimates will cover the vitamin D needs of even those in the population who do not have adequate calcium intakes.

3.4 Part C - Vitamin D-Calcium associations with serum 24,25(OH)$_2$D in a subset of a representative adult population

3.4.1 Specific objective

- To assess the impact of low and high habitual calcium intakes in the absence and presence of low and high serum 25(OH)D status on serum 24,25(OH)$_2$D$_3$ concentration, as an index of vitamin D catabolism and potentially higher requirement, using data and bio-banked serum samples from a subset of adults from the representative National Adult Nutrition Survey (NANS) in Ireland.

Rationale

In the RCT in Part B, relative to >1000 mg/d, calcium intakes <550 mg/d [(5-13% of Irish and UK adults have calcium intakes below 550 mg/d (Henderson *et al*, 2004; IUNA, 2011)] did not influence the response of serum 25(OH)D or serum 24,25(OH)$_2$D$_3$ over winter in older adults. This was a secondary analysis however and numbers of subjects were relatively low (*n* 32 in total with intakes <550 mg/d). Therefore, we used data and biobanked sera from specific subsets of participants in the NANS so as to extend this analysis with greater numbers and in a wider age group (18-84 years). Those NANS participants with calcium intakes below 550 mg/d and with biobanked serum (*n* 64) were matched with those with intakes above 1000 mg/d, and stratified by low and high serum 25(OH)D. Biobanked sera was then used to measure the concentration of 24,25(OH)$_2$D$_3$, as an index of vitamin D catabolism and potentially higher requirement.

3.4.2 Subjects and methods

Subject sampling and recruitment procedures and methods of data collection

A detailed description of the methodology used in the NANS, including the sampling procedure as well as sample recruitment has been reported elsewhere (IUNA 2011; Cashman *et al*, 2013). Briefly, the fieldwork phase of NANS was carried out between October 2008 and April 2010, providing a seasonal balance to the data and biological sample collection. To achieve a nationally representative sample of community-dwelling adults aged 18 years and over, a quota sampling approach was adopted using data from the 2006 Census (Cashman *et al*, 2013). A sample of 1500 free-living adults to represent a population of ~4.2 Million participated in the dietary survey. There were few exclusion criteria, other than pregnancy/lactation and inability to complete the survey due to disability. The study was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals, University College Cork and the Human Ethics Research Committee

of University College Dublin. All eligible and willing participants gave their written consent according to the Helsinki declaration.

Analysis of the demographic features in this sample has shown it to be a representative sample of Irish adults with respect to age, gender, social class and geographical location when compared to Census data (IUNA, 2011; Cashman *et al*, 2013). While participation in the survey did not require provision of a blood sample as an eligibility criteria, all participants were asked if they were willing to provide a blood sample. Of the total group of respondents, 75.5% (n=1132) provided a blood sample. The demographic features of the group of participants who provided a blood sample and those in the entire sample have been described elsewhere (IUNA, 2011; Cashman *et al*, 2013). Seasonality was based on the date the respondents provided the blood sample – November to March (representing the 'winter' period) or April to October (representing the 'summer' period) – consistent with studies based on NHANES in the US and the recent analysis of Canadian Health Measures Survey for vitamin D status.

The approach to food intake data collection, food quantification and estimation of vitamin D and calcium intake in NANS has been provided in detail elsewhere (IUNA, 2011; Cashman *et al*, 2013). Of note, food intake data were analysed using WISP© V3.0 (Tinuviel Software, Anglesey, UK), which uses data from McCance and Widdowson's The Composition of Foods, fifth and sixth editions plus all nine supplemental volumes to generate nutrient intake data, as described elsewhere (IUNA, 2011). Information on social class and education level, smoking status, alcohol intake and, medication usage (including those which contained nutrients) was also collected (IUNA, 2011). Anthropometric measures including height, weight, waist and hip circumference and measures of body composition were taken in the respondents homes, as described previously (IUNA, 2011). The approach towards assessment of vitamin D- and calcium-containing supplement consumption has been provided elsewhere (IUNA, 2011; Cashman *et al*, 2013).

Blood collection

Blood samples were collected by venepuncture into a vacutainer tube by a qualified nurse at designated centres within the survey area or in the respondents' home if the respondent could not travel. Bloods were transported to the laboratory for further processing and serum was stored at -80ºC until required for analysis.

Selection of four subgroups within NANS as required for the present project on impact of dietary calcium on serum 24,25(OH)$_2$D$_3$ concentration

Subjects that fit with the following four subgroupings were identified within the NANS database using existing data on dietary calcium intake estimates and serum total 25(OH)D concentrations:

1. Low calcium intake (<550 mg/d), low serum 25(OH)D (<40 nmol/L)
2. Low calcium intake (<550 mg/d), high serum 25(OH)D (>70 nmol/L)
3. High calcium intake (>1000 mg/d), low serum 25(OH)D (<40 nmol/L)
4. High calcium intake (>1000 mg/d), high serum 25(OH)D (>70 nmol/L)

Subjects within these four subgroupings were broadly matched for sex and age to provide a balance across groups (*n* 31/43 per group).

3.4.2.1 Laboratory analysis

Serum total 25-hydroxyvitamin D

The concentrations of total 25(OH)D (i.e., 25(OH)D$_2$ plus 25(OH)D$_3$) in serum samples for the subgroup of subjects (*n* 141) were measured previously by the Vitamin D Research Group at University College Cork using a liquid chromatography-tandem mass spectrometry (LC-tandem MS) method, described in Part B above and in detail elsewhere (Cashman *et al*., 2014).

Serum 24,25-dihydroxyvitamin D

The concentrations of 24,25(OH)$_2$D$_3$ in serum were measured in bio-banked samples using the liquid chromatography-tandem mass spectrometry (LC-tandem MS) method described in Part B above and in detail elsewhere (Cashman *et al*, 2014).

3.4.2.2 Statistical analysis

Differences in baseline characteristics between genders and between the four groupings were investigated by *t*-tests, ANOVA and chi-square tests, as appropriate. Two-way ANOVA was used to investigate the association between habitual calcium intake and serum 25(OH)D concentration groupings, and their interaction, on serum 24,25(OH)$_2$D$_3$ concentration. Linear regression models were also ran to adjust for potential confounding effects of age, gender and BMI.

3.4.3 Results of Part C

The characteristics of the subsample of adults from NANS used for the present analysis of the associations between dietary calcium, vitamin D status (as reflected by serum 25(OH)D) and serum 24,25$(OH)_2D_3$ concentration are shown in **Table 11**. There was no significant difference between males and females in terms of age, serum 25(OH)D or 24,25$(OH)_2D_3$ concentration (P>0.4 in all cases), males had a borderline (P=0.073) higher calcium intake than females and had a higher mean BMI (P=0.032). The characteristics of the subsample stratified by dietary calcium and serum 25(OH)D status are shown in **Table 12**. The age distribution among the subsample and entire NANS was closely related. The percentage of adults from each of the age categories (18-50 years, 51-64 years and 65+ years) was 67%, 21% and 12%, respectively, in the NANS dataset compared to 66%, 18% and 16%, respectively, in the present subsample. Owing to the need for individuals with calcium intakes below 550 mg/d, there was a higher proportion of females to males (n=84:47) in the present subsample as compared to the entire NANS. There were, however, no significant differences between the four groups of the subsample in terms of proportion of females to males (P=0.3), mean age (P=0.4), mean BMI (P=0.1), or distribution of season of sampling (P=0.07). Even though <500 mg/d and >1000 mg/d calcium intake were used as the threshold bands in the present work, the mean intakes within the groups were 427/463 mg/d and 1232/1391 mg/d for the two low (*low calcium, low serum 25(OH)D* and *low calcium, high serum 25(OH)D*, respectively) and two high calcium intake groups (*high calcium, low serum 25(OH)D* and *high calcium, low serum 25(OH)D,* respectively), respectively.

Two-way ANOVA showed that there was no main effect of calcium intake (<500 mg/d versus >1000 mg/d) on serum 24,25$(OH)_2D_3$ concentration (P=0.6), no significant interaction between calcium intake grouping and serum 25(OH)D concentration grouping (<40 nmol/L versus >70 nmol/L) (P=0.8), but a significant main effect of serum 25(OH)D concentration grouping (P<0.01) (see **Figure 3**). Irrespective of habitual calcium intake grouping, individuals with a high serum 25(OH)D concentration (>70 nmol/L) had a significantly higher mean serum 24,25$(OH)_2D_3$ concentration than individuals with a low serum 25(OH)D concentration (<40 nmol/L) (4.12 ± 1.62 versus 1.23 ± 0.64 nmol/L, respectively; P<0.01). Serum 25(OH)D was strongly correlated with serum 24,25$(OH)_2D_3$ (r=0.76; P<0.0001, n 141). Regression analysis showed that habitual dietary calcium grouping remained a non-significant (P=0.6) determinant of serum 24,25$(OH)_2D_3$ even after accounting for gender, age, BMI and serum 25(OH)D (data not shown).

Table 11. Characteristics of the subsample of adults for the National Adult Nutrition Survey used for the present analysis of associations between dietary calcium intake, vitamin D status and serum $24,25(OH)_2D_3$.[1]

	Total	Men	Women
n	131	47	84
Age	44.0 (17.8)	43.0 (17.5)	44.5 (18.0)
BMI	26.4 (6.1)	28.2 (7.3)	25.2 (4.9)
Dietary Calcium (mg/d)	911 (485)	1033 (488)	842 (473)
Serum 25(OH)D (nmol/L)	55.3 (24.8)	54.1 (24.4)	56.0 (25.2)
Serum $24,25(OH)_2D_3$ (nmol/L)	2.68 (1.90)	2.75 (1.74)	2.64 (1.99)

[1]All values represent Mean (SD)

Table 12. Characteristics of the subsample of adults for the National Adult Nutrition Survey used for the present analysis of associations between dietary calcium intake, vitamin D status and serum 24,25$(OH)_2D_3$ stratified by calcium intake/serum 25(OH)D status.

Grouping:				
(Calcium intake/vitamin D status)	High/Low	High/High	Low/Low	Low/High
Calcium intake threshold	>1000mg/d		<500mg/d	
Serum 25(OH)D threshold	<40nmol/L	>70nmol/L	<40nmol/L	>70nmol/L
n	34	37	31	29
Sex, *n* (%)				
Female	19 (55.9)	22 (59.5)	21 (67.7)	20 (69.0)
Male	15 (44.1)	15 (40.5)	10 (32.3)	9 (24.1)
Age, *n* (%)				
18-50 years	21 (61.8)	24 (64.9)	21 (67.7)	20 (69.0)
51-64 years	8 (23.5)	9 (24.3)	2 (6.5)	5 (17.2)
≥65 years	5 (14.7)	4 (10.8)	8 (25.8)	4 (13.8)
BMI (kg/m^2)[1]	28.6 (7.1)	26.1 (4.1)	24.5 (8.3)	26.4 (4.2)
Season of sampling, *n* (%)				
Extended summer	13 (38.2)	26 (70.3)	16 (51.6)	15 (51.7)
Extended winter	21 (61.8)	11 (29.7)	15 (48.4)	14 (48.3)
Mean calcium intake (mg/d)[1]	1232 (177)	1376 (346)	427 (96)	457 (82)
Mean serum 25(OH)D (nmol/L)[1]	33.0 (5.5)	77.2 (9.2)	30.2 (6.6)	80.6 (8.9)

[1]All values represent Mean (SD)

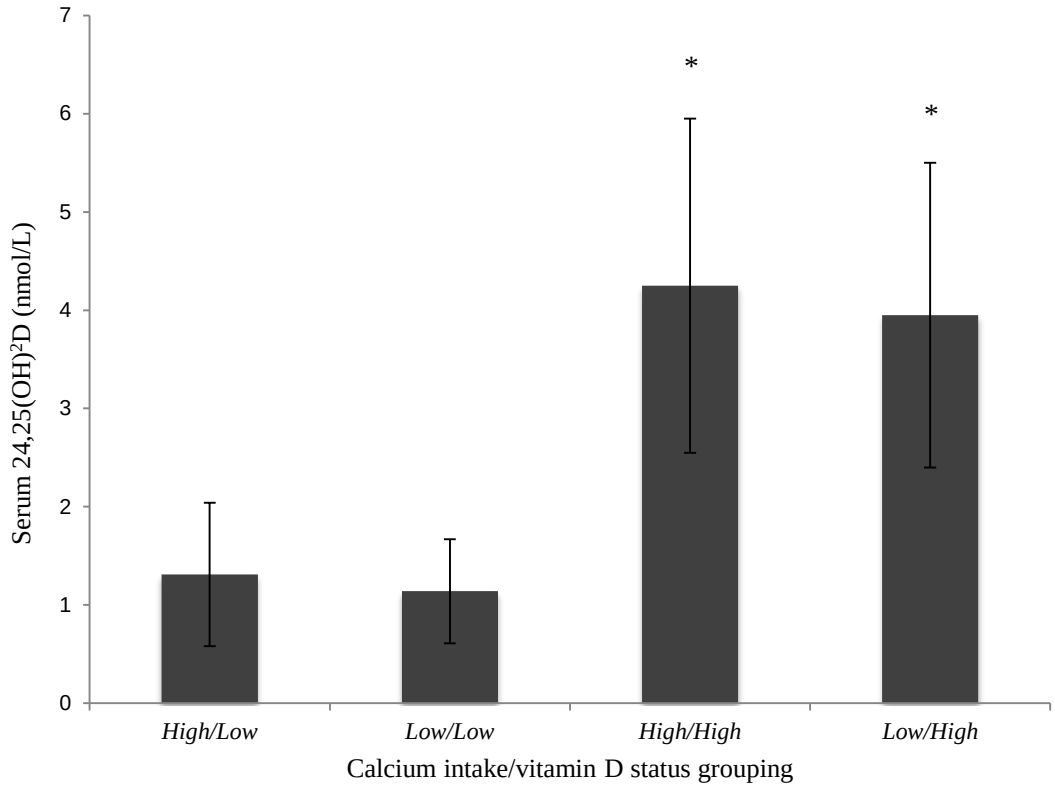

Figure 3. Serum $24,25(OH)_2D_3$ concentration in a subsample of adults for the National Adult Nutrition Survey stratified by dietary calcium intake and serum 25(OH)D grouping. *High calcium intake = >1000 mg/d; low calcium intake = <500 mg/d; High serum 25(OH)D = >70 nmol/L; low serum 25(OH)D = <40 nmol/L. n =31/43 per group.* *Means significantly different from low vitamin D status group within a calcium intake grouping; Two-way ANOVA and Tukey's tests; P<0.01.

3.4.4 Discussion of Part C findings

The finding of this associational analysis which showed that serum 24,25$(OH)_2D_3$, an index of vitamin D catabolism, was unaltered in a subset of adults from a nationally representative sample with habitually low and high calcium intakes, with either low or high serum 25(OH)D concentrations, further support the findings of the new RCT in Part B. Moreover, as the low calcium intake group had habitual intakes below 550 mg/d (and a mean of 445 mg/d, on average), these findings extend and support the secondary analysis of the RCT in which <700 mg/d versus >1000 mg/d was the primary analysis, and <550 mg/d versus >1000 mg/d was the secondary analysis. All subjects with calcium intake below 550 mg/d (and who had an available biobanked serum sample) within the entire NANS were used in the present analysis and thus this result is of public health significance and relevance. Increasing serum 25(OH)D concentration was shown to be the strongest predictor of serum 24,25$(OH)_2D_3$, and in this case unlike the high concentrations of serum 25(OH)D achieved through supplementation with vitamin D_3, the high serum 25(OH)D concentrations were mostly from those subjects sampled in summer-time. Both sets of findings are consistent with the concept that 24-hydroxylation of 25(OH)D represents the first step in the pathway of deactivation when status is good, albeit that there still remains the possibility that 24,25$(OH)_2D_3$ itself may have some biological activity.

3.5. Concluding discussion and remarks

While as per convention the vitamin D DRI was established by the IOM on the assumption that the requirement for dietary calcium is being met (IOM, 2011), a convention followed by most authorities briefed with setting nutrient requirement values, inadequacy in calcium intake could cause changes in the efficient handling of, or physiological response to, vitamin D that might not otherwise be present. A significant portion of adult populations in Europe and North America fail to meet respective dietary calcium requirements (Henderson *et al*, 2004; Flynn *et al*, 2009; IOM, 2011; IUNA, 2011). In fact, the 2010 and 2015 Dietary Guidelines Advisory Committees in the US identified four nutrients of public health concern with calcium being one of them. Thus, clarifying whether low intakes of calcium increase the dietary requirement for vitamin D is important from a public health perspective and also in devising preventative strategies for vitamin D deficiency. This knowledge gap about the influence of calcium intake on the regulation of vitamin D activation and catabolism has been highlighted and prioritized by the IOM (2011) and ourselves (Cashman & Kiely, 2011), as well as forming one of the central basis of the UK Department of Health

research call tenders in which this work was funded in 2012, and which was intended to inform the Vitamin D DRV re-evaluation process in the UK.

While the data from the *post hoc* analyses suggested little, if any, interaction between dietary calcium and vitamin D requirements, it should be acknowledged that none of the four previously-funded vitamin D RCTs were specifically designed to investigate the impact of dietary calcium level and accordingly had relatively small numbers of subjects with calcium intakes which might be considered as being low. To counter this potential limitation the project also conducted a 15 week winter-based vitamin D_3 intervention study in 125 apparently healthy, free-living, white adults aged ≥50 y at a latitude of 51°N in which the potential interactions of dietary calcium intake on both the decline of serum 25(OH)D over winter whilst on a habitual inadequate vitamin D intake as well as on the response of serum 25(OH)D to an intake aimed at achieving at least the IOM suggested 'RDA-like' 25(OH)D concentration of 50 nmol/L (IOM, 2011) were examined. Recent dietary requirement estimates for vitamin D from both sides of the Atlantic have prioritized winter-time as a critical period during which intakes of vitamin D should maintain serum 25(OH)D concentrations above chosen cut-offs (IOM, 2011; German Nutrition Society, 2012; Nordic Nutrition Recommendations, 2012; UK SACN, 2016). A key finding of this new RCT is that the responses in serum 25(OH)D concentrations throughout winter, as well as indices of vitamin D activation and catabolism as potential explanatory variables, were similar in older adults irrespective of whether they were on relatively low (<550/700 mg/d) or high habitual (>1000/1200 mg/d) calcium intakes. Data from NANS also showed a lack of association between calcium intake and serum 24,25$(OH)_2$D in adults aged 18-84 years. Thus, these new data would suggest that recently proposed dietary requirement estimates for vitamin D in North America (IOM, 2011) and then in a number of European member states (German Nutrition Society, 2012; Nordic Nutrition Recommendations, 2012; Weggemans *et al*, 2013; UK SACN 2016) will ensure the adequacy of serum 25(OH)D concentrations in older adults even when the calcium intakes of these adults are in the inadequate range (<700 mg/d, and even <550 mg/d).

It is also interesting to note that when the four new RCT arms (placebo and vitamin D_3 with low habitual calcium intake and placebo and vitamin D_3 with high habitual calcium intake) from the present Part B RCT were included in the RCT database and the meta-regression analysis in Part A was re-performed using with this additional data, there was no overall significant interaction between the response of winter serum 25(OH)D to total vitamin D and dietary calcium category.

These new findings also are important from a policy perspective in terms of devising preventative strategies for vitamin D deficiency. Strategies for bridging the gap between RDA/RNI estimates and current intake of vitamin D in the UK, Ireland, US and other populations (be it dietary advice, and/or supplementation and/or food fortification) will not be dependent on habitual calcium intakes, at least for those with intakes >550 mg/d. While the present study did not include an entire group of subjects with extremely low calcium intakes where perhaps an effect on serum 25(OH)D catabolism may occur, and thus may be a potential limitation, 50% of subjects in the low-moderate calcium intake grouping had intakes <550 mg/d and 25% had intakes <400 mg/d, and all had <700 mg/d. At a population level, only 3% and 5-13% of Irish and UK adults have calcium intakes <400 and <550 mg/d, respectively (Henderson *et al*. 2004; IUNA, 2011).

Likewise, it is possible that the high calcium intake in the present project wasn't high enough to provide the protective effect on serum 25(OH)D. Berlin & Björkhem (1988) reported that supplementation of healthy young adult men (n 14/group) with 2000 mg of calcium per day in addition to the usual diet for 6- to 7-weeks during late autumn/early winter in Sweden led to a significant increase in serum 25(OH)D concentrations (by 30%) relative to that in a control group on a normal diet. The authors also suggest that unpublished data of theirs on an identical trial, but one conducted from February to March, still suggested an elevatory effect of additional calcium on serum 25(OH)D (mean decrease in serum 25(OH)D of 21% versus 9% in control and calcium-supplemented groups, respectively $P<0.002$). Unfortunately, the habitual dietary intake of calcium was not assessed in the study but the authors worked on the assumption that it was about 800 mg/d (Berlin & Björkhem, 1988), which would likely bring their total mean calcium intakes into the range of >2800 mg/d. The upper 2.5 percentile of calcium from all sources in adults in the UK has been reported to be 1794 mg/d and 1550 mg/d for men and women, respectively. Thus, the levels of calcium used by Berlin & Björkhem (1988) are beyond those of a public health relevance, and furthermore two other RCTs found that subjects who had increases in their usual calcium intake of the order of an additional 1000 to 2000 mg of calcium per day (Goussous *et al*, 2005; McCullough *et al*, 2009), likely bringing their total mean calcium intakes into the range of >1500 to >2700 mg/d, did not have a sparing effect on their serum 25(OH)D concentrations over those not supplemented with calcium.

Overall, the data from the present work would suggest dietary requirements for vitamin D are not impacted by calcium intakes, ranging from low to high, and which covers the vast majority of Irish or UK adults. The data will inform the UK and Irish government policy on vitamin D supplementation and fortification of foods. This data will not only be of

importance and relevance to the UK and Ireland, but also internationally as existing vitamin D recommendations/reference intake values are being actively re-evaluated in several European member states as well as in parts of Asia, and possibly elsewhere. The data will also be of interest to the North American IOM in relation to filling one of its identified key knowledge gaps from its recent vitamin D and calcium DRI panel exercise. Thus, the data will contribute to a sound scientific basis for development of nutrition policy and programmes for ensuring adequate vitamin D status in the population.

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Chapter 4

Vitamin D bio-fortification of animal-based foods as a means of increasing the vitamin D intake distribution in the population

Paper in preparation in collaboration with our colleagues in Univeristy College Dublin who performed the feeding trials

4.1 Introduction

Recent data from a pan-European study by Cashman *et al*. (2016) have shown that one in eight Europeans have serum 25-hydroxyvitamin D [25(OH)D] concentrations <30 nmol/L (reflective of vitamin D deficiency) and furthermore, 40% have concentrations less than 50 nmol/L, which the Institute of Medicine (IOM) and others suggest are needed to cover the needs of the majority of the population from a bone health perspective (IOM, 2011; Norden, 2015; EFSA, 2016). While summer ultraviolet B (UVB) sunlight enables cutaneous vitamin D biosynthesis, and thus is a key contributory source of vitamin D for many, excessive exposure is the principal risk factor for the majority of skin cancers, which continue to rise in white populations. Hence, public education campaigns in relation to skin cancer recommend limiting exposure to sunlight. In addition, most European countries experience a vitamin D winter of about 4 to 5 months (Webb *et al*. 2006), during which dermal synthesis of vitamin D is extremely limited due to low UVB availability. Thus, absence of sufficient UVB sunlight for dermal synthesis places increased importance on the vitamin D food supply. As it stands, dietary vitamin D intakes in the population are typically between 3-7 µg/day (Kiely & Black, 2012) and most are not achieving the recently suggested Estimated Average Requirement (EAR) value of 10 µg/day (IOM, 2011). Vinas *et al*. (2011) recently showed that of European national nutrition surveys reporting vitamin D intake data from 2000 onwards, 77-100% and 55-100% of adults (19-64 y) and elderly (>64 y), respectively, had intakes below the EAR. This is not surprising as food sources of vitamin D are few and limited (oily fish, meat, dairy, egg yolk and mushrooms) and many are not consumed on a regular basis (Cashman & Kiely, 2015). In order to improve the dietary supply of vitamin D across the food chain and thus the distribution of intakes in the population, evidence-based, food-based strategies are required.

Bio-fortification of foods with vitamin D is one such strategy that may be employed as an additional approach to traditional fortification practices as a means of closing the gap between current intakes and recommendations. In terms of the present work, bio-fortification refers to the addition of vitamin D and/or 25-hydroxyvitamin D (known commercially as HyD®), where permissible, to livestock feeds with an aim of improving the vitamin D or 25-hydroxyvitamin D contents of the resulting animal produce (e.g., eggs, pork and beef).

Research in this area to date has focused mainly on eggs, but even then the emphasis has been as much on the effect that higher levels of vitamin D compounds in animal feeds may have on the welfare of the animal itself or the quality of their produce (e.g. egg shell strength, laying rate) as on its impact on the vitamin D activity of the resulting eggs. A number of groups have

shown that there is a dose response effect in the vitamin D content of the egg yolk when the diets of laying hens had incremental levels of vitamin D_3 and/or HyD® added (Mattila *et al*., 1999; 2003; 2004; 2011; Yao *et al*., 2013; Browning & Cowieson, 2014). However, several of these studies use levels of inclusion above the upper allowable level for feeds in Europe [3000 IU/kg diet (EFSA, 2005)]. In addition, the studies which used HyD® included it at levels above the upper allowable level [0.080 mg/kg diet (EFSA, 2009)]. Eggs which are produced for human consumption must adhere to these regulations.

In countries where mandatory fortification is not in place, meat can be considered a significant source of vitamin D in the diet (Hill *et al*., 2004). The effect of additional vitamin D_3 and/or HyD® in the diets of pigs and beef cattle, and the resulting vitamin D activity of pork meat and beef, has been much less well investigated and generally where it has been investigated it was again in relation principally to animal welfare and beef tenderness (Swanek *et al*., 1999; Foote *et al*., 2004; Montgomery *et al*., 2004; Sell *et al.*, 2004; Wertz *et al*., 2004; Rivera *et al*., 2005; Cho *et al*., 2006; Lawrence *et al*., 2006; Jakobsen *et al*., 2007; Carnagey *et al*., 2008a; Carnagey *et al*., 2008b; Burild *et al.*, 2016). Even in the two studies by the same group which investigated the effect of additional vitamin D and HyD® in pigs, the results in relation to vitamin D content of pork meat are conflicting (Jakobsen *et al*., 2007, Burild *et al.*, 2016).

Overall, this study aims to investigate the potential for addition of vitamin D and/or HyD® to hen, pig and beef heifer diets, but at levels that do not exceed the respective EU upper levels, on increasing the vitamin D activity of the resulting eggs and meat. Such biofortified foods may protect against vitamin D deficiency in the general population.

4.2 Trials, materials and method

4.2.1 Conduct of layer hen, pig and beef heifer feeding trials at Lyons Research Farm, University College Dublin, Newcastle, Co. Dublin

3.2.1.1 Laying hen feeding trial

A total of sixty laying (Hyline) hens (90% laying), which were receiving standard commercial feeding and management and had a mean initial start weight of 1.68 kg at 25 weeks of age, were randomly assigned to one of four dietary treatment groups (*n* 15 hens/group):

Hen treatment 1 – basal diet with vitamin D_3 (1500 IU/kg);
Hen treatment 2 – basal diet with vitamin D_3 (3000 IU/kg) [EU Upper limit (EFSA, 2009)];
Hen treatment 3 – basal diet with vitamin D_3 (1500 IU/kg) and HyD® (37.5 μg/kg diet; equivalent to 1500 IU/kg feed) [*Total equivalent, 3000 IU/kg feed]*;
Hen treatment 4 – basal diet with HyD® (EFSA, 2009) (75 μg/kg diet; equivalent to 3000 IU/kg feed).

The experimental diets were based on a basic diet containing crude protein, 155 g/kg, and metabolizable energy, 10.44 MJ/kg. Diets were also balanced for amino acid profiles and fatty acid content, and were provided in a layer mash form. The vitamin D_3 and HyD® for inclusion in the experimental diets in this hen trial, and also the pig feeding trial (see Section 3.2.1.2 below), were provided by DSM Nutritional Products Ltd, Ayrshire, UK. Each experimental feed was produced at the beginning of the feeding trial in 100 kg batches. The vitamin D_3 and 25-hydroxyvitamin D_3 contents of the experimental diets were determined independently by the Danish Technical University (DTU), Denmark, using a modified liquid chromatography tandem mass spectrometry (LC-MS/MS) method (Jakobsen *et al.,* 2004, 2007). The DTU laboratory has been accredited for vitamin D and 25-hydroxyvitamin D since 1994 and 2004, respectively. For the last 15 years, it has performed the analysis for vitamin D and 25-hydroxyvitamin D in food for the Danish and Norwegian Food Composition Tables.

The hens were housed in a barn system throughout the experiment. All treatment groups received a 14-hour lighting pattern, with free access to ten laying boxes per house. All houses were bedded in wood shavings; and all treatment groups had adequate perching and scratching space. Birds were fed at a rate of 140 g/per head/day with *ad libitum* supply of water at all times. Water was supplied in round drinkers and hens had adequate space allowance of 2.5 cm/hen at drinkers.

Eggs from the four different treatment groups were collected on two separate days (days 1 and 3) during the last week of the study (week 6). Eggs were transported to the Food Processing Hall, University College Cork (UCC) for processing and storage before vitamin D and sensory analysis. The vitamin D_3 and 25-hydroxyvitamin D_3 content of individual (*n* 6-8 eggs) as well as pooled egg yolk samples (pool of 16 eggs) from each treatment group were analysed by the *Cork Centre for Vitamin D and Nutrition Research,* UCC using an adapted high performance liquid chromatography (HPLC) method (See Section 3.2.3 below). A pooled egg yolk sample (based on pool of 16 eggs) from each of the dietary treatment groups was sent to DTU, Denmark for external analysis of vitamin D compounds by a modified LC-MS/MS method (Jakobsen *et al*., 2004, 2007).

4.2.1.2 Pig feeding trial

Twenty four (Large white x Landrace) finisher pigs, which were receiving standard commercial feeding and management and with a mean initial starting weight of 60 kg, were blocked on the basis of live weight and sex, and allocated at random to one of three dietary treatment groups (*n* 8 pigs/group):

Pig treatment 1 – basal diet with vitamin D_3 (2000 IU/kg) [Upper EU limit] (EFSA, 2012);
Pig treatment 2 – basal diet with HyD® (EFSA, 2009) (50 μg/kg diet; equivalent to 2000 IU/kg feed);
Pig treatment 3 – basal diet and vitamin D_3 (1000 IU/kg) and HyD® (25 μg/kg diet; equivalent to 1000 IU/kg feed) *[Total equivalent, 2000 IU/kg feed]*.

The experimental diets were based on wheat, barley and soya-bean meal. Diets were fed in meal form and formulated to have identical contents of energy (13.8 MJ/kg) and total lysine (9.5 g/kg). Each diet was made at the beginning of the experiment in two x 5 tonne batches. Feed samples were taken at manufacture and throughout the experimental period for chemical analysis. The vitamin D_3 and 25-hydroxyvitamin D_3 contents of the experimental diets were determined independently by DTU (as per Section 3.2.1.1 above).

The pigs were penned in mixed groups of twelve (six males and six females) and were stocked at 1.0 m^2 per pig per treatment. The house was mechanically ventilated to provide an ambient temperature of 18°C. Each pen had a solid floor lying area with access to slats at the rear. Individual single space computerised feeders with water nipples were present in all pens providing *ad libitum* supply of both food and water at all times. Animals were individually tagged and weighed on days 1, 14, 28 and 42 of the experiment. All pigs were removed on day 66 of the experiment for slaughter, at which stage the pigs had live weights of ≥90 kg.

The pigs were slaughtered and processed to meats at Rosderra Irish Meats Group, Co. Offaly, which is the largest pork processing company in Ireland.

Blood samples were obtained at slaughter from the *vena jugularis*, collected in BD Vacutainer® blood collection tubes, and processed to serum within 4 hours at Lyons Research Farm, Newcastle, Co. Dublin. Serum was frozen at -80°C until analysis. Serum total 25-hydroxyvitamin D [25(OH)D] concentrations were measured by the *Cork Centre for Vitamin D and Nutrition Research,* UCC using a certified LC-MS/MS method (see Section 3.2.2 below).

At 24-hours *post-mortem*, samples of loin and fillet were harvested and transported to the Food Processing Hall, UCC for further processing and storage. Upon arrival, sub samples of loin and fillet from each animal in each treatment group were collected, packaged according to treatment group, and stored at 4°C prior to preparation of pooled samples. The remainder of the selected cuts from each animal were individually vacuum packed and labelled accordingly. Individual samples were then stored at -20°C for future analysis. The following day, the aforementioned sub samples were minced and further mixed using a Buchi blender to ensure homogeneity of the pooled sample. This was carried out by treatment group, with adequate cleaning of equipment between different treatment groups. The pooled sample was then sub-divided into aliquots for both DTU and UCC. Pooled samples from each treatment group were stored at -80°C, for future analysis of vitamin D compounds in UCC and prior to transport to DTU. Pooled samples of both loin and fillet (based on meat samples from 8 pigs) from each treatment group were transported on dry ice to DTU, Denmark for independent analysis of vitamin D_3 and 25-hydroxyvitamin D_3 (as per Section 3.2.1.1). Individual samples of loin and fillet from each animal in the treatment groups were also stored for meat quality and sensory analysis at UCC.

4.2.1.3 Beef cattle feeding trial

Thirty continental beef heifers (Charolais and Limousin cross), with a mean initial weight 600 kg, were blocked on the basis of breed and body weight, and randomly allocated to one of three dietary treatment groups (*n* 10 cattle/group):

Beef treatment 1 – basal diet with no vitamin D_3 added;
Beef treatment 2 – basal diet with vitamin D_3 (2000 IU/kg feed);
Beef treatment 3 – basal diet with vitamin D_3 (4000 IU/kg feed) [current EU upper limit] (EFSA, 2012).

The basal diet consisted of a high-energy, concentrate finishing ration, which was offered to heifers in conjunction with their respective dietary treatments on an *ad libitum* basis. Animals were also offered straw as a long fibre source. All dietary treatments had a constant forage:concentrate ratio. The vitamin D_3 for inclusion in the experiments were provided by DSM Nutritional Products Ltd (as per Section 3.2.1.1 above). Treatments were offered for the final three weeks of an 80-day finishing period. Each treatment diet was manufactured at the beginning of the experiment in two batches. Feed samples were taken at manufacture and throughout the experimental period for chemical analysis. The vitamin D_3 and 25-hydroxyvitamin D_3 contents of the experimental diets were determined independently by DTU (as per Section 3.2.1.1 above).

For the duration of the 3-week experimental feeding period, the animals were housed indoors under normal lighting conditions (i.e., no UVB), with no access to the outside. Individual feed intakes were recorded daily using a Calan gate feeding system, where feed was weighed in and refusals weighed back to calculate daily feed intakes and dry matter intakes. Heifers were weighed on day 0, 7, 14, 21 and day 28 of the experiment to calculate average daily gains.

Prior to slaughter, on day 80 of the finishing period, blood samples were collected by a veterinarian and processed to serum at Lyons Research Farm, Newcastle, Co. Dublin. Sera were stored at -80°C until transport to UCC for subsequent analysis of serum 25(OH)D concentrations. The animals were slaughtered at Kildare Chilling, Co. Kildare, a long-standing Irish beef and lamb processing plant. At 48-hours *post-mortem*, primal cuts were harvested and transported to the Food Processing Hall, UCC for processing and storage. The meat samples were stored at 4°C before the selected cuts of rib-eye were boned and trimmed by a craft butcher. Sub-samples of rib-eye from each animal (*n* 8) in each treatment group were minced and further mixed using a Buchi blender to ensure homogeneity of the pooled sample. Pooled samples (based on meat samples from 7 heifers per pool) were transported on dry ice to DTU, Denmark for independent analysis of vitamin D_3 and 25-hydroxyvitamin D_3 contents (as per Section 3.2.1.1). Individual and pooled samples from each treatment group were stored at -20°C and -80°C, respectively, for future analysis of vitamin D compounds in UCC. Individual meat samples from each animal were also stored for meat quality and sensory analysis at UCC.

4.2.2 Measurement of serum 25(OH)D concentrations

The concentrations of total 25(OH)D (i.e., 25(OH)D_2 plus 25(OH)D_3) in the porcine and bovine serum samples were measured by the *Cork Centre for Vitamin D and Nutrition Research* at UCC using a certified LC-MS/MS method, as has been described in detail elsewhere (Cashman *et al*., 2013). In brief, the LC-MS/MS method measures 25(OH)D_2 and 25(OH)D_3 in serum as well as the 3-epimer of 25(OH)D_3 (3-epi-25(OH)D_3), which is not chromatographically resolved from 25(OH)D_3 by most routine LC-MS/MS methods. The presence of 3-epimers of 25(OH)D can pose problems for LC-MS/MS methods because the precursor ion and fragmentation patterns are the same as 25(OH)D, thus failure to account for these metabolites can result in overestimation of 25(OH)D_3 in particular as the quantitatively more abundant metabolite. The intra-assay CV of the method was <5% for all 25-hydroxyvitamin D metabolites, while the inter-assay CV was <6%. The *Cork Centre for Vitamin D and Nutrition Research* is a participant in the VDSP (Sempos *et al*. 2012) and is certified by Centers for Disease Control and Prevention's Vitamin D Standardization Certification Program (Rahmani *et al*. 2013). The inter-assay CV for total 25(OH)D was 3.6%. Both the VDSP and the certification program reports total 25(OH)D as well as 25(OH)D_2 and 25(OH)D_3 using the higher order reference laboratories. In addition, the quality and accuracy of human serum total 25(OH)D analysis by the LC-MS/MS in our laboratory is monitored on an ongoing basis by participation in the Vitamin D External Quality Assessment Scheme [DEQAS, Charing Cross Hospital, London, UK].

All solvents and mobile phase additives were MS grade and purchased from Sigma-Aldrich (Wicklow, Ireland). Zinc sulphate was sourced from Sigma-Aldrich while stable isotope labelled d_3-25(OH)D_2, d_3-25(OH)D_3 and d_3-3-epi-25(OH)D_3 were purchased from Isosciences (4667 Somerton Road, Trevose, PA 19053, USA). Certified calibrators for 25(OH)D_2 and 25(OH)D_3 were bought from the National Institute of Standards and Technology (NIST), USA (SRM 2972) while a CertiMass reference standard for 3-epi-25(OH)D_3 was sourced from Isosciences. Low and High serum QC materials were commercially available from Chromsystems (Munich, Germany). The chromatographic column was a Supelco Ascentis Express F5 available from Sigma-Aldrich.

4.2.3 Determination of vitamin D compounds and total activity in pork, beef and egg yolk

4.2.3.1 Egg yolk sample preparation (Pooled and individual)

Whole eggs were stored for a maximum of two weeks in the cold storage room (4°C) at the Food Processing Hall, UCC prior to sample preparation. The whole egg was weighed using a standard balance. Egg yolk and albumin (white) were separated. The egg yolk and wet eggshell were weighed individually. Egg albumin weight was determined using the following equation:

$$Albumin\ (g) = whole\ egg\ (g) - [wet\ egg\ shell\ (g) + egg\ yolk\ (g)]$$

The weight of each component was recorded for individual egg samples. Individual egg yolks were added to plastic containers (Sarstedt, 50 ml, polypropylene container) that were labeled accordingly, blanketed with nitrogen gas and stored at -80°C until analysis.

Egg yolks were pooled according to dietary treatment. Pooled samples were added to plastic containers that were labeled accordingly, mixed to ensure homogeneity, blanketed with nitrogen gas and stored at -80°C until analysis or transport to DTU, Denmark.

4.2.3.2 Measurement of vitamin D compounds and total activity in pooled egg yolk and meat samples via LC-MS/MS at DTU, Denmark

Vitamin $D_{2/3}$ and 25(OH)$D_{2/3}$ content of pooled egg yolk, pork and beef samples were analysed in DTU, Denmark using a modification of a sensitive LC-MS/MS method, as described in detail elsewhere (Jakobsen *et al*, 2004, 2007).

The vitamin D analysis performed at DTU on pooled samples was in order to expedite a decision on which of the various treatment might be used in further feeding trials for ultimate supply of eggs for a randomised controlled trial in Irish adults (see Chapter 5). However, this pooled analysis only provided very limited data on the variability around the mean vitamin D and metabolite content. Therefore, analysis of vitamin D and 25(OH)D was conducted on individual egg samples from the various treatment groups at UCC using an in-house HPLC method (see Section 3.2.3.3 below). The data from this method was aligned with that from the DTU method to ensure comparability.

4.2.3.3 Measurement of vitamin D metabolites in pooled and individual egg yolk and meat samples via HPLC at UCC

All work was carried out under amber light where possible. Vitamin D and 25-hydroxyvitamin D were purchased from Sigma-Aldrich Ireland Ltd. (Arklow, Ireland). Heptane, 2-propanol, acetonitrile and methanol were HPLC grade. All other reagents were analytical grade.

Saponification: Raw egg yolk (individual sample) or approximately 16 g of pooled egg yolk sample (representative of average yolk weight) was thawed at room temperature and transferred into appropriately labeled 250 ml Erlenmeyer flasks. Internal standards were added using a calibrated pipette, 50 µl 10 µg/ml vitamin D_2 and 25 µl of 10 µg/ml 25-hydroxyvitamin D_2. Ethanol (100 ml) was added to all samples. Ascorbic acid was prepared freshly on the day (10% w/v) and 20 ml of this solution and 50 ml of KOH (50%, w/v) were added to each flask and mixed. All samples were blanketed with nitrogen, sealed with parafilm and saponified overnight on a magnetic stirring block.

Extraction: The extraction procedure was adapted from Jakobsen *et al.*, (2004). In brief, the contents of the Erlenmeyer flask were transferred into 500 ml separating funnels and extracted with diethyl ether (130 ml) and petroleum ether (130 ml). The extract was washed 3 times by inversion with 50 ml of ultrapure water. Residual water was removed from the extract by adding a small amount of anhydrous sodium sulphate. The extract was evaporated on a rotary evaporator until approx. 5 ml remained. The residue was transferred to a small tube and the remaining ether evaporated under nitrogen. The residue was reconstituted in 5 ml of heptane. Solid phase extraction was carried out using Mega Bond-Elut silica columns (2 g) (Agilent Technologies); Samples were dried in a vacuum centrifuge (miVac Quattro concentrator, Genevac) and reconstituted in 1.5 ml of 2-propanol (1%) in heptane. The extract was then transferred to a centrifugal spin filter (0.45 µm nylon membrane) and centrifuged at 2500 rpm for five minutes before the semi-preparative HPLC stage.

Standards: Standards for semi-preparative HPLC were prepared from solid vitamin D_3 and 25-hydroxyvitamin D_3. Stock solutions were prepared in heptane (1 mg/ml), stored at -20°C and diluted as required for retention time checks. Working standard solutions (10 µg/ml) for analytical HPLC were prepared from certified solutions of vitamin D_3 (1 mg/ml ethanol) and 25-hydroxyvitamin D_3 (100 µg/ml) and stored at -80°C. Concentrations were checked every six months by measuring the absorbance of stock solutions at 265nm. The molar extinction coefficients used were 18,466 (D_3) and 18,584 ($25OHD_3$). Calibration curves were prepared in acetonitrile:methanol (90:10) (20-200 ng/ml vitamin D_3) or methanol:water (50:50) (3-50 ng/ml 25-hydroxyvitamin D_3).

HPLC: The same HPLC system was used for semipreparative and analytical HPLC (Shimadzu Corporation, Kyoto, Japan). The system consisted of two LC20ADXR pumps, SIL-30AC autosampler, CTO-20AC column oven, SPD-30MA PDA detector, FRC-10A fraction collector and CBM-20A system controller. Labsolutions Lite software was used for system control and data acquisition and processing. Semi-preparative steps were based on the methods of Jakobsen *et al*., (2004, 2007). The first semi-preparative step used two columns in series (Lichrospher Si-60, 5µm, 250mm x 4.6mm i.d.; Lichrospher Amino, 5µm, 150 x 4.6mm i.d., both supplied by Sigma-Aldrich Ireland Ltd.). Gradient elution using 2-propanol (solvent A) and n-heptane (solvent B) was as follows: 96% solvent B for 10 min.; 92% B for 15 min.; 96% B for 10 min. The flow rate was 1 ml/min. The collected vitamin D and 25OHD fractions were further purified using a cyano column (Supelco LC-CN, 5um, 150mm x 4.6mm i.d.). The mobile phase was 2-propanol:heptane (1:99) at 1 ml/min. Vitamin D was analysed using a Vydac 201TP C18 column (250 x 3mm i.d., 5µm, Grace Davison Discovery Sciences, Hesperia, California, USA). The mobile phase was acetonitrile:methanol (90:10) at a flow rate of 0.25 ml/min. Column temperature was 30°C. 25-Hydroxyvitamin D_3 was analysed using an Ascentic Express C18 column (100 x 4.6mm i.d., 2.7µm, Sigma-Aldrich Ireland Ltd.). The mobile phase was methanol:water (86:14) at a flow rate of 1ml/min. Column temperature was 45°C. The injection volume was 45 µl. The PDA detector was set to scan from 200-500nm and spectra were extracted at 265nm. Concentrations were calculated based on external calibration and adjusted for percent recovery of the internal standard. Following correction, results were within 92% of expected values for vitamin D_3 and 25-hydroxyvitamin D_3.

In all cases, total vitamin D activity was calculated as the content of vitamin D_3 plus the content of 25(OH)D_3 × 5, which is the calculation used in McCance & Widdowson's, food composition tables. The conversion factor of 5 is consistent with the findings from our randomized controlled trial of older adult Irish men and women which showed that supplemental 25-hydroxyvitamin D_3 was 5 times more potent in raising winter serum 25(OH)D of compared with an equivalent amount of supplemental vitamin D_3 (Cashman *et al*. 2012).

4.2.4 Sensory analysis of vitamin D-enhanced eggs, pork and beef

4.2.4.1 Preparation and sensory evaluation of eggs for sensory trials

Boiled eggs

Whole eggs were placed in a saucepan of cold water and boiled for 10 minutes on a conventional cooker hob. Following cooking, eggs were cooled in cold water, the shells were removed and each boiled egg was cut in half (longitudinally) prior to presentation to sensory panelists (see below).

Fried eggs

Eggs were cracked open and cooked (5 minutes in vegetable oil) within stainless steel cooking rings placed in a frying pan, to ensure uniformity and consistency of fried eggs for sensory evaluation. Fried eggs were cut in half and re-heated for 20 seconds in a microwave prior to presentation to sensory panelists (see below).

Sensory evaluation of eggs

Sensory evaluation of boiled and fried eggs was carried out in two separate sensory analysis sessions using 18-22 naïve assessors where four egg samples (one from each dietary treatment), identified with random three digit codes, were presented to each panelist in duplicate. Sample presentation order was randomised to prevent any flavour carryover effects. Sensory analysis was undertaken in the panel booths at the sensory laboratory, UCC, in accordance with ISO (1988) international standard regulations. Assessors were also provided with water and crackers to cleanse their palates between samples.

Hedonic (appearance, liking of flavour, liking of texture and overall acceptability) (dislike/like, unacceptable/acceptable) and intensity sensory analysis descriptors (yolk colour (yellow/orange), egg white (white/grey), sulphur flavour (none/extreme), sour (not/very), sweet (not/very), salty (not/very), odour (weak/strong) and off-flavour (none/extreme)) were determined where assessors were asked to indicate their rating on a 10 centimetres (cm) continuous line scale ranging from 0 to 10. Results for sensory analysis scores were measured in cm.

4.2.4.2 Preparation and sensory evaluation of pork loin for sensory trials

Pork loin slices (2.5 cm in thickness) from each dietary treatment were covered with aluminium foil and cooked in an oven (Zanussi Professional, Model 10 GN1/1, Conegliano, Italy) at 180ºC until an internal meat temperature of 72ºC was reached. Following cooking and cooling, pork was cut into 2 cm x 2 cm cubes, pooled for each treatment group and identified with random three-digit codes. Sample presentation order was randomised to prevent any flavour carryover effects (MacFie *et al.*, 1989). Prior to serving to panellists, pork samples were re-heated in a microwave for 20 seconds to release the meat odour and flavour.

Sensory evaluation of pork loin

Sensory analysis of cooked pork loin was performed in duplicate by a total of 20 naïve assessors, as described by O'Sullivan *et al*. (2003). Sensory analysis was undertaken in the panel booths at the sensory laboratory, UCC in accordance with the ISO (1988) international standard regulations. Assessors were provided with water to cleanse their palates between samples.

Hedonic sensory analysis descriptors were appearance, odour, liking of texture, liking of flavour and overall acceptability. Off-flavour was selected as an intensity sensory analysis descriptor. Assessors were asked to indicate their opinions on a 10 cm continuous line scale ranging from 0 (extremely dislike / none) to 10 (extremely like / extreme). Results for sensory analysis scores were measured in cm.

4.2.4.3 Preparation and sensory evaluation of beef steaks for sensory trials

Beef steaks from each dietary treatment were covered with aluminium foil and cooked in an oven (Zanussi Professional, Model 10 GN1/1, Conegliano, Italy) at 200ºC until an internal meat temperature of 72ºC was reached. Following cooking, steaks were cooled, cut into 2 cm x 2 cm cubes, pooled for each treatment group and identified with random three-digit codes. Sample presentation order was randomised to prevent any flavour carryover effects (MacFie *et al.*, 1989). Prior to serving to panellists, beef samples were re-heated in a microwave for 20 seconds to release the meat odour and flavour.

Sensory evaluation of beef steaks

Sensory analysis of cooked beef steaks was performed in duplicate by a total of 40 naïve assessors over two analysis days, as described by O'Sullivan *et al*. (2003). Sensory analysis was undertaken in the panel booths at the sensory laboratory, UCC in accordance with the ISO (1988) international standard regulations. Assessors were provided with water to cleanse their palates between samples.

Hedonic sensory analysis descriptors were appearance, odour, liking of texture, liking of flavour and overall acceptability. Off-flavour was selected as an intensity sensory analysis descriptor. Assessors were asked to indicate their opinions on a 10 cm continuous line scale ranging from 0 (extremely dislike / none) to 10 (extremely like / extreme). Results for sensory analysis scores were measured in centimetres (cm).

4.2.5 Statistical analysis

Data and statistical analysis on egg and meat (pork and beef) vitamin D_3, 25- hydroxyvitamin D_3 and total vitamin D activity as well as serum 25(OH)D concentrations for pigs and beef cattle was conducted using SPSS© Version 22.0 for Windows™ (SPSS Inc. Chicago, IL, USA). Data was tested for normality and Ln transformed as required (serum 25(OH)D_2 in pigs). Differences in mean serum 25(OH)D variables and vitamin D_3, 25- hydroxyvitamin D_3 and total vitamin D activity across groups were analysed by one-way ANOVA followed by Tukey's *post hoc* tests.

Statistical analysis of the sensory score data, via analysis of variance (ANOVA)-partial least squares regression (APLSR), was conducted using Unscrambler software (Version 10.3, CAMO ASA, Trondheim, Norway). Statistical significance was defined as a $P < 0.05$. For the pork loin and beef steak sensory evaluation, in order to investigate the predictive ability of the dietary treatment received on the sensory score outcome, the X-matrix was designated as 0/1 for the dietary treatment and the Y-matrix designated as the sensory descriptors. To derive significance indications for the relationships determined in the quantitative APLSR, regression coefficients were analysed by jack-knifing which is based on cross-validation and stability plots (Martens & Martens, 2001). Results were presented as significance of regression coefficients. From these, the significances ($P<0.05$) of the variable relationships in the X- and Y- matrices were determined.

4.3. Results

4.3.1. Vitamin D content of vitamin D-enhanced eggs

The vitamin D_3, 25-hydroxyvitamin D and total vitamin D activity in the pooled raw egg yolk samples derived from egg collections from hens fed four experimental diets are shown in **Table 1**. Within treatment groups, estimates for all three vitamin D-related measures were variable across the two pools made from two separate collection days (d1 and d3) of the same week. For example, the percentage difference in egg vitamin D_3 content, 25-hydroxyvitamin D content, and vitamin D activity results ranged from 10.4-41.0%, 0.4-22.4%, and 9.7-24.0%, respectively. Thus, data from the pools from both days were combined to provide an average for each treatment group, and the means of each vitamin D content-related measure were compared across groups. One-way ANOVA showed that vitamin D_3, 25-hydroxyvitamin D and total vitamin D activity in the combined pools differed by treatment ($P<0.05$, in all cases).

The vitamin D_3 contents of raw egg yolk pools in the three 3000 IU/kg treatment groups were similar to that in the 1500 IU/kg group. Within the three 3000 IU/kg treatment groups, the vitamin D_3 content of egg yolk pools from the 3000 IU vitamin D_3/kg group were significantly ($P=0.022$) higher than that from the 3000 IU HyD®/kg group; with no other significant differences observed. The 25-hydroxyvitamin D_3 contents were significantly higher in the 3000 IU combination group (1500 IU vitamin D_3 + 1500 IU HyD®/kg) and the 3000 IU HyD®/kg group in comparison to the 1500 IU/kg group ($P<0.05$, in both cases). Within the 3000 IU/kg equivalent groups, the 3000 IU vitamin D_3/kg group contained significantly lower 25-hydroxyvitamin D_3 than the other two treatment groups ($P<0.05$, in both cases). The vitamin D activity of the pooled egg sample from the 3000 IU HyD®/kg group was significantly higher than the 1500 vitamin D_3/kg group ($P=0.05$). No other significant differences were observed between treatment groups.

Total vitamin D activity was prioritized as it takes account of vitamin D_3 and 25-hydroxyvitamin D as well as fact that the latter is 5 times more effective in raising serum 25(OH)D than the former. The eggs from hens fed the 3000 IU HyD®/kg diet had the highest numerical mean total vitamin D activity, followed by the 3000 IU combination group, 3000 IU vitamin D_3/kg group and the 1500 IU vitamin D_3/kg group, in that order. However, it should be stressed that the mean total vitamin D activity in the 3000 IU HyD®/kg egg pool was not statistically different to that found in the egg pools from the other 3000 IU equivalent treatment groups ($P>0.05$), whereas it was significantly higher than that of the 1500 IU vitamin D_3 group.

Table 1. Vitamin D_3, 25-hydroxyvitamin D_3 and total vitamin D activity of pooled egg yolk samples from eggs collected from hens fed the four experimental vitamin D diets: as determined via LC-MS/MS at the Danish Technical University.

Egg vitamin D content	Treatment groups [additional vitamin D compounds/kg feed]				*P*-value[2]
	1500 IU vitamin D_3	*3000 IU vitamin D_3*	*3000 IU combination*[1]	*3000 IU HyD®*	
	µg/100g raw egg yolk				
Vitamin D_3:					
Pooled Day 1	7.85	13.44	9.14	4.12	-
Pooled Day 3	4.63	10.96	7.27	3.69	-
Mean (SD)	6.24 (2.28)	12.20 (1.75)[a]	8.21 (1.32)[ab]	3.91 (0.30)[b]	0.026
25-hydroxyvitamin D_3:					
Pooled Day 1	2.48	2.31	4.92	5.85	-
Pooled Day 3	2.15	2.32	3.82	5.29	-
Mean (SD)	2.32 (0.23)	2.32 (0.01)[a]	4.37 (0.78)[*,b]	5.57 (0.40)[*,b]	0.005
Total vitamin D activity:[3]					
Pooled Day 1	20.2	25.0	33.7	33.4	-
Pooled Day 3	15.4	22.6	26.4	30.1	-
Mean (SD)	17.8 (3.4)	23.8 (1.7)	30.1 (5.2)	31.8 (2.3)[*]	0.047

HyD® =25-hydroxyvitamin D

n per treatment = 16 egg yolks/pool for day 1 and also for day 3.

[1]3000 IU combination = 1500 IU vitamin D_3 + 1500 IU HyD®

[2]One–way ANOVA followed by Tukey's; *Significant differences ($P \leq 0.05$) between group means of 1500 IU/kg diet and the three different 3000 IU/kg diet groups; [a,b]Different supercript letters represent significant ($P \leq 0.05$) differences among group means of three groups receiving 3000 IU/kg diet.

[3]Total vitamin D activity calculated as vitamin D_3 plus 25-hydroxyvitamin D_3 × 5 (Cashman *et al*., 2012)

The vitamin D_3, 25-hydroxyvitamin D and total vitamin D activity in eggs from hens fed four experimental diets, based on individual analysis of eggs (*n* 6-8/group) rather than pooled samples, are shown in **Table 2**. One-way ANOVA showed that mean vitamin D_3, 25-hydroxyvitamin D and total vitamin D activity in the groups of individual eggs differed by treatment ($P<0.001$ in all cases).

The 1500 IU vitamin D_3/kg and 3000 IU HyD®/kg treatment groups produced eggs with similar vitamin D_3 contents; the vitamin D_3 content of eggs from these groups were significantly lower than eggs produced from either the 3000 IU vitamin D_3 or 3000 IU combination groups ($P<0.01$, in both cases). The 3000 IU vitamin D_3 group produced eggs with the highest vitamin D_3 content of all groups ($P<0.01$, in all cases). The 25-hydroxyvitamin D_3 contents were significantly higher in eggs from the 3000 IU combination group and 3000 IU HyD® group than the eggs produced from the 2 groups with additional vitamin D_3 only ($P<0.01$, in all cases). The 25-hydroxyvitamin D_3 content of eggs from the 3000 IU combination group and 3000 IU HyD® group were not statistically different from each other ($P>0.1$). Eggs from the 3000 IU HyD® had higher total vitamin D activity than eggs from the 1500 IU vitamin D_3 group ($P=0.0001$); whereas eggs from the 3000 IU combination group were statistically higher than eggs from both of the additional vitamin D_3 only groups ($P<0.01$). The percentage coefficient of variation (CV) for vitamin D activity in each of the treatment groups were 18.4%, 12.3%, 14.7% and 27.6% for 1500 IU vitamin D_3, 3000 IU vitamin D_3, 3000 IU combination, and 3000 IU HyD®, respectively.

In terms of vitamin D activity, eggs from the 3000 IU combination and 3000 IU HyD® groups were not statistically different from each other; however, individual egg analysis showed that the eggs from hens fed the 3000 IU combination diet had the highest numerical mean total vitamin D activity, followed by the 3000 IU HyD®/kg group, 3000 IU vitamin D_3/kg group and the 1500 IU vitamin D_3/kg group in that order.

Table 2. Vitamin D_3, 25-hydroxyvitamin D_3 and total vitamin D activity of individual eggs (*n* 6-8/group) collected from hens fed the four experimental vitamin D diets: as determined via HPLC at University College Cork[1]

Egg vitamin D content…	Treatment groups [additional vitamin D compounds/kg feed]				*P*-value[3]
	1500 IU vitamin D_3	*3000 IU vitamin D_3*	*3000 IU combination*[2]	*3000 IU HyD®*	
	µg/100g raw egg yolk				
Vitamin D_3	3.0 (1.6)	10.2 (2.3)^{*,a}	6.7 (0.9)^{*,b}	2.6 (1.7)^{c}	<0.0001
25-hydroxyvitamin D_3	3.0 (0.8)	3.2 (0.8)^{a}	6.7 (1.1)^{*,b}	6.2 (2.1)^{*,b}	<0.0001
Total vitamin D activity[4]	18.1 (3.4)	26.1 (3.3)^{a}	40.4 (6.0)^{*,b}	33.4 (9.2)^{*,ab}	<0.0001

HyD® =25-hydroxyvitamin D

[1]Values represent means (SD).

[2]3000 IU combination = 1500 IU vitamin D_3 + 1500 IU HyD®

[3]One–way ANOVA followed by Tukey's; *Significant differences ($P \leq 0.01$) between group means of 1500 IU/kg diet and the three different 3000 IU/kg diet groups; ^{a,b,c}Different supercript letters represent significant ($P \leq 0.01$) differences among group means of three groups receiving 3000 IU/kg diet.

[4]Total vitamin D activity calculated as vitamin D_3 plus 25-hydroxyvitamin D_3 × 5 (Cashman *et al*., 2012)

4.3.2 Sensory analysis of fried and boiled eggs across vitamin D dietary treatment groups

The average sensory scores of boiled and fried eggs from each vitamin D dietary treatment group are shown in **Table 3** and **4**, respectively. There were no significant positive or negative correlations between vitamin D dietary treatments and the sensory descriptors in resulting eggs when boiled (Table 3) or fried (Table 4) (P <0.05).

4.3.3. Serum 25(OH)D concentration and total vitamin D content of meat from pigs and beef heifers from vitamin D treatment groups

Serum 25(OH)D concentrations and the vitamin D_3 and 25-hydroxyvitamin D_3 contents of pork meat based on pooled (n 7/8 per group) and individual analysis (n 7/group) from pigs fed the three different vitamin D experimental diets are outlined in **Table 5**. Serum total 25(OH)D and 25(OH)D_3 concentrations were significantly different across the treatment groups (P=0.001, for both). Serum 25(OH)D_3 and total 25(OH)D concentrations (see **Figure 1**) were significantly higher (P=0.001) in pigs that received the 2000 IU HyD® compared to those in the 2000 IU vitamin D_3 group, but no other group differences were observed. There were no significant differences (P>0.4) in serum 25(OH)D_2 concentrations between the treatment groups.

The vitamin D_3 contents of the pork meat were significantly different between the three different treatment diet groups. The vitamin D_3 content of the pork meat was highest from pigs fed the 2000 IU vitamin D_3 diet, followed by the 2000 IU combination diet and the 2000 IU HyD® diet, respectively. Within the three different diet treatment groups, the 25-hydroxyvitamin D_3 content of the pork meat was significantly higher in pigs fed the 2000 IU HyD® diet in comparison to the 2000 IU vitamin D_3 group (P = 0.001); with no other significant differences observed. In terms of the vitamin D activity of the pork meat, pork meat from the group that was fed 2000 IU HyD diet, had the highest total vitamin D activity, followed by the 2000 IU combination group and the 2000 IU vitamin D_3 group in that order.Vitamin D activity of the pork meat was significantly higher in pigs fed the 2000 IU HyD® diet in comparison to the 2000 IU vitamin D_3 group (P = 0.01); with no other significant differences observed .

Table 3. Average sensory scores (cm) for boiled eggs from each dietary treatment; values represented as mean (S.D.) Samples were presented in duplicate (*n* 22)[1]

Treatment group	Hedonic				Intensity							
	Appearance	*Liking of Flavour*	*Liking of texture*	*Overall acceptability*	*Yolk colour*	*Egg white*	*Sulphur flavour*	*Sour*	*Sweet*	*Salty*	*Odour*	*Off flavour*
					Yellow/ orange	*White/grey*	*None/ extreme*	*Not/very*	*Not/very*	*Not/very*	*Weak/ strong*	*None/ extreme*
1500 IU vit D_3	6.70 (1.13)	6.73 (1.20)	6.88 (1.20)	6.90 (0.93)	3.80 (1.84)	3.73 (1.81)	2.40 (1.89)	1.15 (1.21)	2.03 (1.70)	1.69 (1.53)	3.43 (1.77)	1.25 (1.19)
3000 IU vit D_3	6.19 (1.39)	6.25 (1.40)	6.52 (1.39)	6.36 (1.11)	3.81 (1.77)	3.83 (1.84)	2.44 (1.68)	1.22 (1.25)	2.27 (1.75)	1.78 (1.93)	2.76 (1.34)	1.21 (1.34)
3000 IUcombo[2]	6.69 (1.41)	6.77 (1.13)	7.16 (1.14)	6.89 (0.81)	4.76 (1.96)	3.52 (1.80)	2.13 (1.68)	1.22 (1.25)	2.11 (1.97)	1.90 (1.99)	3.32 (1.80)	1.10 (1.36)
3000 IU HyD®	6.93 (1.51)	6.52 (1.34)	6.76 (1.77)	6.83 (1.30)	4.30 (2.23)	3.44 (2.00)	2.31 (1.720	1.22 (1.54)	2.23 (1.83)	1.77 (1.87)	2.72 (1.72)	1.20 (1.46)

HyD® =25-hydroxyvitamin D

[1]*P*>0.05 for all.

[2]3000 IU combo = 1500 IU vitamin D_3 + 1500 IU HyD®

Table 4. Average sensory scores (cm) for fried eggs from each dietary treatment; values represented as mean ± S.D. Samples were presented in duplicate (*n* 18)[1]

Treatment group	Hedonic				Intensity							
	Appearance	*Liking of Flavour*	*Liking of texture*	*Overall acceptability*	*Yolk colour*	*Egg white*	*Sulphur flavour*	*Sour*	*Sweet*	*Salty*	*Odour*	*Off flavour*
					Yellow/ orange	*White/grey*	*None/ extreme*	*Not/very*	*Not/very*	*Not/very*	*Weak/ strong*	*None/ extreme*
1500 IU vit D_3	6.61 (1.38)	6.73 (1.17)	5.91 (1.40)	6.40 (1.35)	4.78 (1.92)	2.69 (0.98)	1.38 (1.21)	0.76 (0.89)	1.89 (1.85)	1.71 (1.31)	2.78 (1.22)	0.44 (0.78)
3000 IU vit D_3	6.35 (1.64)	6.59 (1.44)	5.89 (1.50)	6.29 (1.37)	4.75 (2.11)	2.68 (1.49)	0.88 (0.77)	0.91 (1.03)	1.61 (1.79)	1.47 (1.52)	2.77 (1.60	0.75 (1.30)
3000 IUcombo[2]	6.47 (1.23)	6.53 (1.18)	6.26 (1.06)	6.36 (1.04)	5.30 (2.40)	2.64 (1.19)	0.93 (1.33)	0.92 (1.50)	1.93 (1.92)	1.47 (1.39)	2.55 (1.75)	0.87 (1.49)
3000 IU HyD®	6.54 (1.46)	6.52 (1.17)	6.54 (1.33)	6.76 (1.20)	4.79 (2.26)	2.41 (1.40)	0.77 (0.89)	0.84 (1.07)	1.86 (1.91)	1.20 (1.74)	2.49 (1.88)	0.75 (1.22)

HyD® =25-hydroxyvitamin D

[1]*P*>0.05 for all.

[2]3000 IU combo = 1500 IU vitamin D_3 + 1500 IU HyD®

Table 5. Serum 25(OH)D concentrations and content of vitamin D_3 and 25-hydroxyvitamin D_3 in pork meat from pigs (*n* 7/8 per group) fed the three experimental vitamin D diets[1]

	Treatment group [Additional vitamin D compounds/kg feed]			
	2000 IU vitamin D_3	*2000 IU combination*[2]	*2000 IU HyD®*	*P-value*[7]
Porcine serum (nmol/L)[3]:				
25(OH)D_2	0.08 (0.20)	<LOD*	0.42 (0.48)	0.403
25(OH)D_3	69.3 (12.8)[a]	104.9 (32.9)[ab]	147.1 (46.6)[b]	0.001
Total 25(OH)D	69.4 (12.9)[a]	104.9 (32.9)[ab]	147.5 (46.9)[b]	0.001
Meat (μg/100g)				
Pooled (n 8)[4]				
Vitamin D_3	0.17 (0.01)	0.09 (0.01)	0.03 (0.01)	-[8]
25(OH)D_3	0.14 (0.00)	0.22 (0.02)	0.33 (0.02)	-
Total vitamin D activity [5]	0.89 (0.00)	1.17 (0.09)	1.70 (0.12)	-
Individual (n 7) [6]				
Vitamin D_3	0.20 (0.02)[a]	0.12 (0.04)[b]	0.04 (0.02)[c]	< 0.0001
25-hydroxyvitamin D_3	0.13 (0.03)[a]	0.18 (0.05)[ab]	0.29 (0.09)[b]	0.001
Total vitamin D activity [5]	0.87 (0.19)[a]	1.03 (0.27)[ab]	1.49 (0.44)[b]	0.01

HyD® =25-hydroxyvitamin D
[1]Values represent mean (S.D.)
[2]2000 IU combination = 1000 IU vitamin D_3 + 1000 IU[1] 25(OH)D_3
[3]Determined via LC-MS/MS at University College Cork; Total 25(OH)D = 25(OH)D_2 + 25(OH)D_3.
[4]Determined via LC-MS/MS at Danish Technical University; representing combined data from pooled samples of loin and fillet cuts (each containing an *n* 8)
[5]Total vitamin D activity = vitamin D_3 + 25(OH)D_3 × 5
[6]Determined via LC-MS/MS at University College Cork; representing data from individual samples (*n* 7)
[7]One–way ANOVA followed by Tukey's; [a,b] Groups with different superscript letters are statistically different from each other; *P*=0.001
[8]These data did not undergo a statistical comparison, as they are pooled data.
*Below the limit of detection (LOD) and for statistical analysis the LOD/square root of 2 was used.

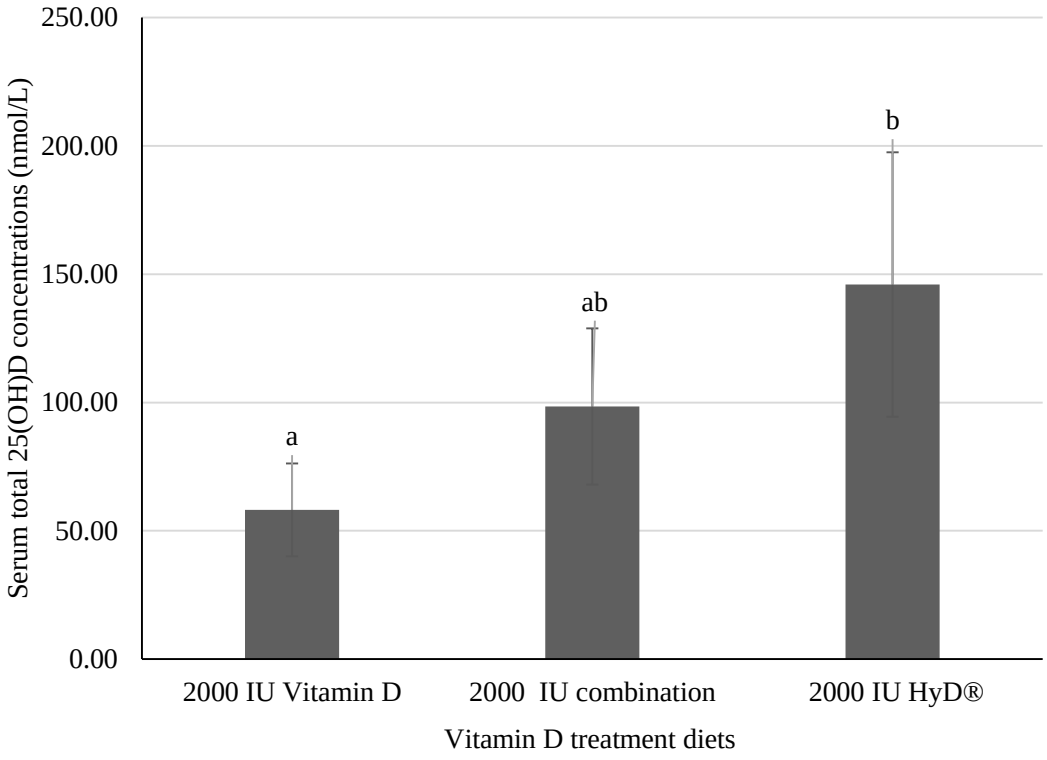

Figure 1. Serum total 25(OH)D concentrations (nmol/L) for pigs fed the three dietary vitamin D experimental diets. Bars and error bars represent mean (SD (*n* 7/ 8 per group). Bars with different lower case letters are significantly different to each other (P = 0.001; via ANOVA and Tukey's). '2000 IU combination' represents the treatment diet of 1000 IU vitamin D_3 + 1000 IU HyD®*;* HyD® = 25-hydroxyvitamin D.

Serum 25(OH)D concentrations and the vitamin D_3 and 25-hydroxyvitamin D_3 contents of beef based on pooled and individual analysis (*n* 7/group) from heifers fed the three different vitamin D experimental diets are outlined in **Table 6**. Serum total 25(OH)D and 25(OH)D_3 concentrations were significantly different across the treatment groups (P<0.001, for both). Serum 25(OH)D_3 and total 25(OH)D concentrations (see **Figure 2**) were significantly higher (P<0.05, for both) in heifers that received the 2000 and 4000 IU vitamin D_3/kg compared to those in no vitamin D_3 group, with no differences between the 2000 and 4000 IU vitamin D_3 groups (P>0.05). There were no significant differences (P>0.9) in serum 25(OH)D_2 concentrations between the treatment groups.

The vitamin D_3 and 25- hydroxyvitamin D_3 contents of the beef were significantly different between the three different treatment diet groups (P < 0.0001). The vitamin D_3 content of the beef was highest in the group fed 4000 IU vitamin D_3/ kg, followed by the 2000 IU- and 0 IU vitamin D_3/kg groups, respectively. Likewise, the 25-hydroxyvitamin D content was highest in the beef from heifers that received the 4000 IU vitamin D_3/ kg, followed by the 2000 IU- and 0 IU vitamin D_3/kg groups, respectively. In terms of the vitamin D activity of the beef, beef from heifers fed the 4000 IU vitamin D_3 /kg diet had the highest total vitamin D activity followed by the 2000 IU vitamin D_3/ kg and the 0 IU vitamin D_3/ kg group in that order..4.3.4 Sensory analysis of pork loin across vitamin D dietary treatment groups

The mean sensory scores for pork loin from each vitamin D dietary treatment group are shown in **Table 7**. The regression coefficients as shown in **Table 8** were derived from jack-knife uncertainty testing (as described in section 3.2.5). No significant differences existed across the vitamin D dietary treatment groups for any of sensory descriptors.

Table 6. Serum 25(OH)D concentrations and content of vitamin D_3 and 25-hydroxyvitamin D_3 in tissue from beef heifers (*n* 7/group) fed the three experimental vitamin D_3 diets[1]

	Treatment group [Additional vitamin D_3/kg feed]			
	0 IU vitamin D_3	*2000 IU vitamin D_3*	*4000 IU vitamin D_3*	*P-value*[5]
Bovine serum (nmol/L)[2]				
25(OH)D_2	22.1 (9.9)	21.4 (6.7)	20.9 (6.1	0.960
25(OH)D_3	67.5 (8.9)[a]	118 (12.8)[b]	158 (50.3)[b]	<0.001
Total 25(OH)D	89.5 (15.7)[a]	139 (18.7)[b]	179 (52.7)[b]	<0.001
Meat (μg/100g)				
Pooled[3]				
Vitamin D_2	<0.1	<0.1	<0.1	-[6]
Vitamin D_3	0.019	0.045	0.067	-
25(OH)D_2	0.024	0.027	0.022	-
25(OH)D_3	0.083	0.168	0.172	-
Total vitamin D activity	0.55	1.02	1.04	-
Individual[4]				
Vitamin D_3	0.01(0.01)[a]	0.04 (0.01)[b]	0.10 (0.02)[c]	<0.0001
25-hydroxyvitamin D_3	0.11 (0.02)[a]	0.18 (0.02)[b]	0.25 (0.05)[c]	<0.0001
Total vitamin D activity	0.55 (0.09)[a]	0.95 (0.10)[b]	1.35 (0.28)[c]	<0.0001

HyD® =25-hydroxyvitamin D

[1]values represent mean (S.D).

[2]Determined via LC-MS/MS at University College Cork; Total 25(OH)D = 25(OH)D_2 + 25(OH)D_3.

[3]Representing data from pooled samples rib-eye (each containing an *n* 7); determined via LC-MS/MS at Danish Technical University; Total vitamin D activity = vitamin D_2 + D_3 + [25(OH)D_2 × 5] + [25(OH)D_3 × 5]*;* no vitamin D_2 estimates included as below level of quantification.

[4]Representing data from individual samples (*n* 7); determined via LC-MS/MS at University College Cork; Total vitamin D activity = vitamin D_3 + [25(OH)D_3 × 5]

[5]One–way ANOVA followed by Tukey's; [a,b] Groups with different superscript letters are statistically different from each other; $P<0.005$

[6]These data did not undergo a statistical comparison, as they are pooled data.

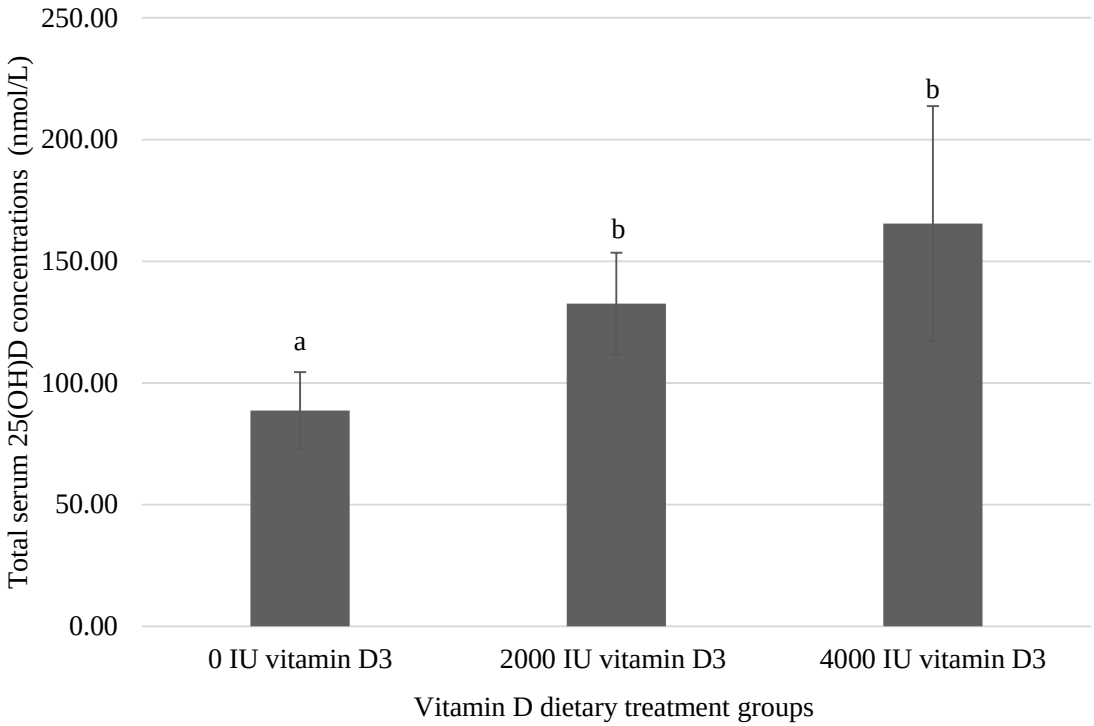

Figure 2. Serum total 25(OH)D concentrations (nmol/L) for beef heifers fed the three different vitamin D experimental diets. Bars and error bars represent mean (SD) (*n* 10, 11 per group). Bars with different lower case letters are significantly different to each other ($P<0.05$; via ANOVA and Tukey's).

Table 7. Average sensory scores (cm) for pork loin from each dietary treatment; values represented as mean (S.D.) Samples were presented in duplicate (*n* 20)

Treatment group	*Appearance*	*Odour*	*Texture*	*Flavour*	*Overall acceptability*	*Off-flavour*
2000 IU vitamin D_3	6.41 (1.86)	6.06 (1.95)	5.37(2.34)	5.48 (2.00)	5.92 (1.83)	2.08 (2.41)
1000 IU vitamin D_3 + 1000 IU HyD®	5.94 (2.37)	6.28 (1.64)	6.09 (2.49)	6.15 (1.99)	6.38 (1.85)	1.66 (2.15)
2000 IU HyD®	6.18 (1.97)	6.04 (1.97)	5.48 (2.76)	5.32 (2.18)	5.56 (2.06)	2.31 (2.59)

Table 8. Regression coefficients from the analysis of variance (ANOVA)-partial least squares regression analysis of pork loin, as derived by jack-knife uncertainty testing

Treatment group	*Appearance*	*Odour*	*Texture*	*Flavour*	*Overall acceptability*	*Off-flavour*
2000 IU vitamin D_3	0.555	-0.415	-0.469	-0.250	-0.206	0.271
1000 IU vitamin D_3 + 1000 IU HyD®	-0.441	0.327	0.437	0.141	0.156	-0.153
2000 IU HyD®	0.367	-0.354	-0.339	-0.131	-0.194	0.210

No significance ($P > 0.05$)

4.3.5 Sensory analysis of beef steak across vitamin D dietary treatment groups

The average sensory scores for beef steak from each vitamin D dietary treatment group are shown in **Table 9**. The regression coefficients as shown in **Table 10** were derived from jack-knife uncertainty testing (as described in section 3.2.5). Overall acceptability was found to be significantly lower for beef steaks from the 0 IU treatment diet group in comparison to beef steaks from the treatment groups that received additional vitamin D_3 via jack knife testing. There were no significant differences in overall acceptability between the 2000 IU and 4000 IU vitamin D_3 dietary treatment groups. No further significant differences existed across the vitamin D dietary treatment groups for the remaining sensory descriptors.

Table 9. Average sensory scores (cm) for beef steak from each dietary treatment; values represented as mean (S.D.) Samples were presented in duplicate (*n* 40)

Treatment group	*Appearance*	*Odour*	*Texture*	*Flavour*	*Overall acceptability*	*Off-flavour*
0 IU vitamin D_3	6.18 (1.84)	5.84 (1.85)	5.47 (2.36)	5.88 (2.06)	5.72 (2.04)	1.50 (2.15)
2000 IU vitamin D_3	6.46 (1.84)	6.10 (1.94)	6.07 (2.27)	6.17 (2.08)	6.42 (1.76)	1.71 (2.24)
4000 IU vitamin D_3	6.52 (2.14)	6.29 (1.96)	6.00 (2.23)	6.35 (1.86)	6.40 (1.82)	1.26 (1.84)

Table 10. Regression coefficients from the analysis of variance (ANOVA)-partial least squares regression analysis of beef steaks, as derived by jack-knife uncertainty testing

Treatment group	*Appearance*	*Odour*	*Texture*	*Flavour*	*Overall acceptability*	*Off-flavour*
0 IU vitamin D_3	-0.134	-0.062	-0.093	-0.139	-0.024*	0.982
2000 IU vitamin D_3	0.974	0.624	0.352	0.931	0.178	-0.337
4000 IU vitamin D_3	0.769	0.624	0.531	0.380	0.233	-0.221

*indicates significance at the (P<0.05) level.

4.4 Discussion

The low dietary vitamin D intakes in European populations, as outlined comprehensively by Vinas *et al.* (2011), highlights the fact that the current food supply (even including current food fortification and vitamin D supplementation practices) are not enough to prevent wide-scale low vitamin D status in Europe (Cashman *et al*. 2016). This is particularly the case during the months of the year where UVB availability is inadequate for dermal synthesis of vitamin D (Webb *et al*., 2006) and where individuals follow recommendations to limit summer sun exposure for the prevention of skin cancer (NICE, 2016). The present work has focused on bio-fortification of eggs, pork and beef with vitamin D as a method of improving the dietary availability of vitamin D from the current food supply and in an effort to reduce the discrepancy that exists between typical dietary intakes of vitamin D and recently established recommendations.

Eggs are a widely consumed food and therefore potentially an ideal vehicle to target as a vitamin D-biofortified food. Previous studies have demonstrated the effectiveness of additional vitamin D_3 and/or HyD® in the feed of laying hens in terms of improving the vitamin D_3 and 25-hydroxyvitamin D contents of the resulting eggs (Mattila *et al*., 1999; 2003; 2004; 2011; Yao *et al*., 2013; Browning & Cowieson, 2014). However, the majority of these studies have included levels of vitamin D and/or HyD® in the feed which exceed the EU allowable maximum levels for use in laying hen feed (equivalent to 3000 IU/kg feed; EFSA, 2009, EFSA, 2012). **Figure 4** below shows the data on vitamin D_3 content of the eggs from these published studies, and has also included data from the current study for reference. Studies which used feed levels of vitamin D and/or HyD® in excess of the maximum allowable levels are shown in the graph using red bars, those which have used permissible levels are shown using blue bars and the present study is indicated using the green bars. Likewise, **Figure 5** below shows available data on 25-hydroxyvitamin D content of the eggs from these published studies, and has also included data from the present study. Prior to the present study, data on vitamin D, and especially the metabolite, content of eggs from hens provided with feed containing permissible levels of vitamin D and/or HyD® were limited and, moreover, the available data were quite variable (Figure 4 and 5). This is particularly the case for 25 hydroxyvitamin D data in eggs (Figure 5), and yet knowledge of the 25-hydroxyvitamin D content is vital in determining the total vitamin D activity (i.e., which accounts for the vitamin D plus 25-hydroxyvitamin D by a factor of 5; Cashman *et al*. 2012) of the biofortified eggs.

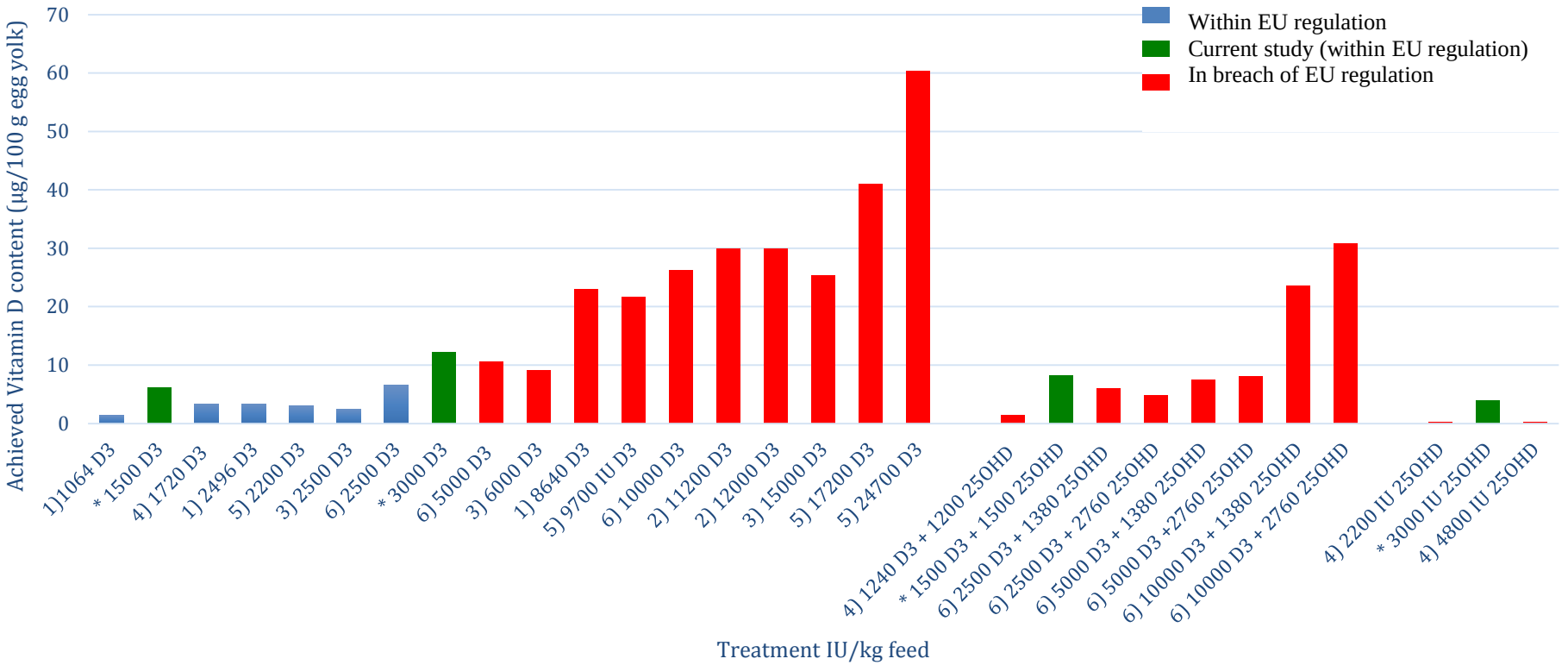

Figure 4. The achieved vitamin D_3 content of egg yolk (µg/100g) reported from published studies of the effect of different vitamin D and/or HyD® supplementation in laying hen feeds. The first number in parenthesis in the X axis denotes the study from which this data is derived as follows: 1) Mattila *et al*., 1999; 2) Mattila *et al*., 2003; 3) Mattila *et al*., 2004; 4) Mattila *et al*., 2011; 5) Yao *et al*., 2013; 6) Browning & Cowieson, 2014. *Pooled results from the current study (as determined via LC-MS/MS at DTU) are presented in the graph.

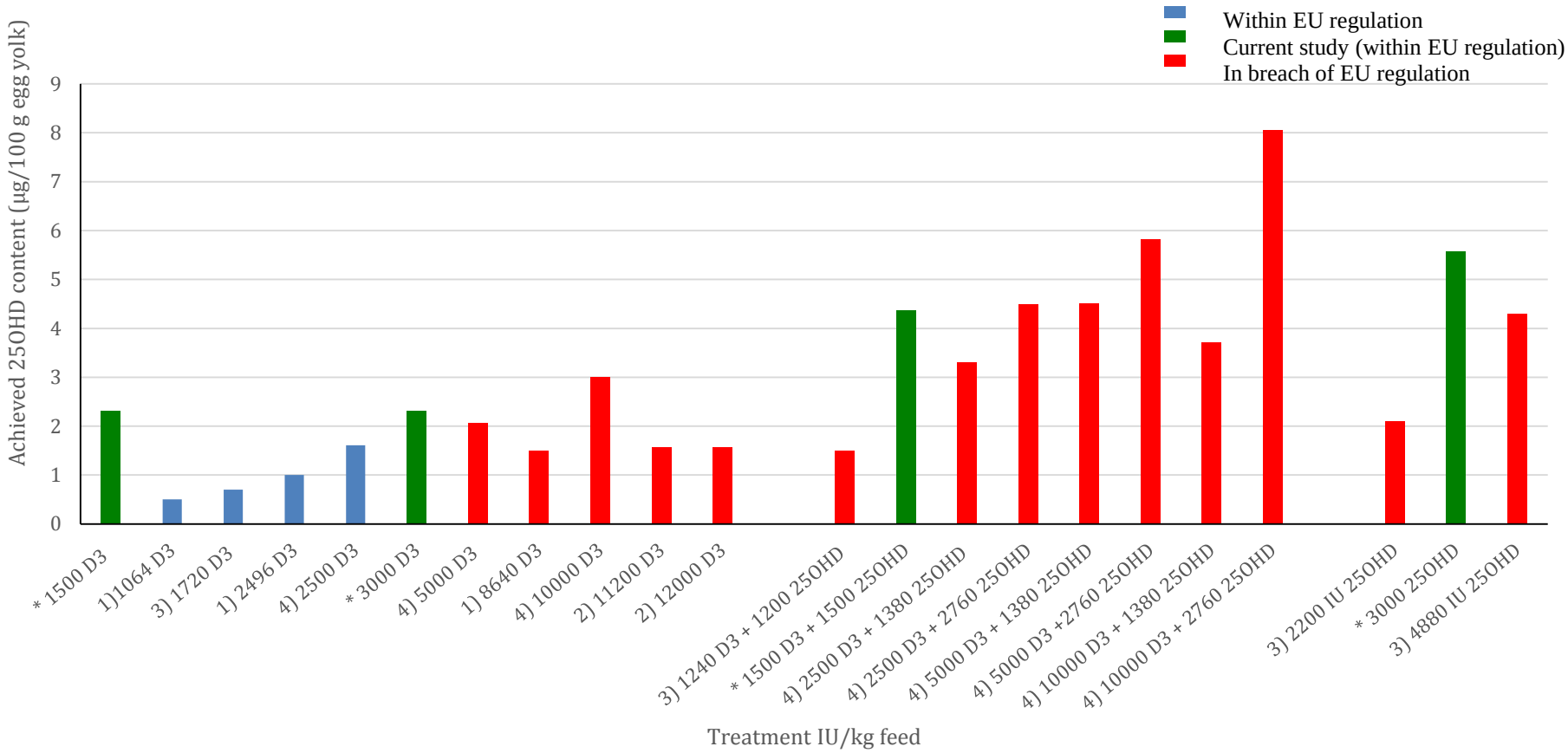

Figure 5. The achieved 25-hydroxyvitamin D content of egg yolk (µg/100g) reported from published studies of the effect of different vitamin D and HyD® supplementation in laying hen feeds. The first number in parenthesis in the X axis denotes the study from which this data is derived as follows: 1) Mattila et al., 1999; 2) Mattila et al., 2003; 3) Mattila et al., 2011; 4) Browning & Cowieson, 2014. *Pooled results from the current study (as determined via LC-MS/MS at DTU) are presented in the graph.

In general, the measurement of 25 hydroxyvitamin D in food is limited due to the small number of research groups who undertake its measurement. In addition, even in the laboratories that undertake this analysis, the variability in estimates of 25 hydroxyvitamin D in food can be large. For example, the inter-lab coefficient of variation (CV) for vitamin D_3 and 25 hydroxyvitamin D_3 analysis of whole egg powder by five different labs participating in an inter-laboratory trial was 10% and 16%, respectively. The equivalent CVs for vitamin D_3 and 25 hydroxyvitamin D_3 analysis of ground beef was 8% and 24%, respectively (Personal communication from Jette Jakobsen, DTU). This situation will be helped by the fact that the National Institute of Standards and Technology (NIST) in the US will soon include a value for 25 hydroxyvitamin D content in some food standard reference materials. This needs to be borne in mind when making comparisons of vitamin D content or total activity in eggs coming from different studies, even when the levels of vitamin D compound included in the hen feeds are comparable. The present study used the National Food Institute, Technical University of Denmark for the principal vitamin D_3 and 25 hydroxyvitamin D analysis, and then the data from our in house HPLC was aligned with that method to ensure comparability. The National Food Institute laboratory has been accredited for vitamin D and 25-hydroxyvitamin D since 1994 and 2004, respectively. For the last 15 years, it has performed the analysis for vitamin D and 25-hydroxyvitamin D in food for the Danish and Norwegian Food Composition Tables.

Mattila *et al*. (2011), in a feeding trial similar to the present one, used levels of vitamin D and HyD® for inclusion in the hen feeds which were allowable by EU regulations, except for one high dose HyD® level (at 4800 IU HyD®/kg feed). The other three levels of vitamin D and/or HyD® used by Mattila *et al*. (2011) (i.e., 1720 IU vitamin D/kg feed; 1240 IU vitamin D and 1200 IU HyD®/kg feed; and 2200 IU HyD® /kg feed) are lower than that used in our study which limits a direct comparison of final egg vitamin D and 25-hydroxyvitamin D contents from the two studies. Of note, however, Mattila *et al*. (2011) found that hens receiving additional HyD® alone produced eggs with a very low vitamin D_3 content (<0.2 µg/100 egg yolk), despite a clear increase in 25-hydroxyvitamin D content. In contrast, our present findings show that hens receiving additional HyD® alone (and albeit at slightly higher levels 3000 IU/kg feed versus 2200 IU/kg in Mattila *et al*. 2011 study) had relatively good vitamin D_3 content (3.9 µg/100 egg yolk) as well as the expected increase in 25-hydroxyvitamin D content. This also translated into total vitamin D activity being much higher in our HyD®-biofortified eggs compared to that in the Mattila *et al*. (2011) study (~5 v. ~2 µg/whole egg, respectively). A key difference between the two studies, besides being analysed in different laboratories, is that the hens in the Mattila *et al*. (2011) study were provided with HyD® in their feeds throughout their laying lifespan, whereas in our study they received HyD®-containing feed for 6 weeks of the experimental diet period, following consumption of a

typical commercial diet for several months prior to that. This may have facilitated the higher vitamin D of the egg, unlike that in Mattila *et al*. (2011) study. Thus, it may be that use of HyD® in hen diets should be used in a fashion almost akin to a 'finisher' style diet in heifers, so as to achieve optimal content of both vitamin D compounds in the egg.

Studies on the sensory acceptability of vitamin D_3-biofortified eggs to date have been on eggs in which the hens received well above the European allowable upper level (Mattila *et* al., 2003, Yao *et al*., 2013), and HyD® eggs have not been tested. The findings of our study suggest that consumer acceptability of eggs from hens fed 3000 IU vitamin D_3/kg diet or 3000 IU HyD®/kg diet did not differ from each other, or from hens fed the 50:50 vitamin D_3:HyD® diet (equivalent to 3000 IU/kg) or control diet (3000 IU vitamin D_3/kg). That the HyD®- or vitamin D_3-derived eggs had equal consumer acceptability profiles based on the present sensory evaluation was important, as any alteration to a sensory perception of a food that may be deemed negative by the consumer will affect their decision to consume the product.

Overall, the findings of our study suggest that the addition of vitamin D and/or HyD® at levels, that adhere to the EU maximum allowable levels set by EFSA, can increase the total vitamin D activity of an egg to ~5 µg/whole egg, whilst maintaining good consumer acceptability profiles. Thus, from a content perspective, these vitamin D-biofortified eggs can provide about half of the EAR value of 10 µg/day (IOM, 2011). Of key importance in terms of informing the potential of vitamin D-biofortified eggs as a food-based solution for prevention of vitamin D deficiency, is the need to test and illustrate the effect of consumption of such biofortified eggs on vitamin D status of healthy human subjects in a randomized controlled trial setting, which is the focus of Chapter 5.

In terms of the evidence-base for vitamin D-biofortified pork, there have been a number of pig studies (Wiegand *et al*., 2002; Swigert *et al*., 2004; Wilborn *et al*., 2004) which included vitamin D_3 in the feed but at supra-nutritional levels, that exceed the current EU maximum allowable level of 2000 IU/kg feed (EFSA, 2009). Three studies have reported the effect of inclusion of vitamin D and/or HyD® in pig feedstuffs, at or below the current EU allowable levels, on vitamin D_3 and 25-hydroxyvitamin D content of pork meat (Jakobsen *et al*. 2007, Höller *et al*. 2010, Burild *et al*. 2016). Burild *et al*. (2016) have stressed an important consideration in comparing absolute levels of vitamin D compounds in pork meat from these different studies, due to differences in study design and experimental animals, but also importantly analytical methods. As mentioned above, variability among labs in the assessment of vitamin D_3, but 25-hydroxyvitamin D in particular, is considerable.

In a study design very similar to the present pig study, Jakobsen *et al*. (2007) reported that while use of vitamin D_3 or HyD® alone, or both in combination (all at *circa* 2000 IU equivalent/kg feed), for 16 weeks prior to slaughter led to minimal differences in plasma 25(OH)D concentrations in pigs, there was a clear dose-related increments in vitamin D_3 content of lean pork (loin) meat with increasing vitamin D_3 level in the three feeds (i.e., from 0 to 1000 to 2000 IU/kg in the HyD®, 50:50 vitamin D_3:HyD® and vitamin D_3 feed, respectively). The 25-hydroxyvitamin D content of pork meat was variable, with no difference between pigs fed the vitamin D_3 alone and HyD® alone feeds, but was significantly higher in pigs fed the vitamin D_3+HyD® feed (Jakobsen *et al*. 2007). This led the authors to suggest that HyD® in pig feed leads to meat with a lower vitamin D nutritional value compared to feeds containing vitamin D_3. While not reported in the paper, comparison of total vitamin D activity (using the meat vitamin D and metabolite data and a factor of 5 for the 25-hydroxyvitamin D) of pork meat from animals fed HyD® or vitamin D_3 (2000 IU per kg diet) showed only a slight difference (4.1 versus 5.6 μg/kg meat, respectively). More recently, however, another pig study from the same Danish group showed that serum 25(OH)D concentration, and the contents of vitamin D_3 and 25-hydroxyvitamin D in lean pork (loin) meat, all increased in a linear manner in pigs fed increasing levels of vitamin D_3 in feeds (5-50 μg/kg) over 7 weeks prior to slaughter (Burild *et al*. 2016). Similarly, while serum 25(OH)D concentration and the 25-hydroxyvitamin D content of lean pork meat increased in a linear manner in pigs fed increasing levels of HyD® in the feeds (5-50 μg/kg diet), the increase in pork meat vitamin D_3 content was relatively weak, if not absent altogether (Burild *et al*. 2016). Again while not reported in the paper, comparison of total vitamin D activity (again using the meat vitamin D and metabolite data and a factor of 5 for the 25-hydroxyvitamin D) of pork meat from animals fed HyD®- or vitamin D_3-containing feed (2000 IU per kg) showed a two-fold higher activity in the former compared to the latter (14.2 versus 6.5 μg/kg meat, respectively). Likewise, serum 25(OH)D concentration was over twice as high in the animals fed the HyD®- versus vitamin D_3-containing feed (67.1 v. 27.2 ng/mL, respectively) (Burild *et al*. 2016). These agree well with the findings of the present study which showed that serum 25(OH)D concentration and total vitamin D activity of lean pork (loin and fillet) meat from pigs fed HyD® was double that from pigs fed vitamin D_3, whereas the pigs provided with feeds containing 50:50 HyD®:vitamin D_3 had intermediate serum 25(OH)D concentrations and total vitamin D activity in pork meat.

The total vitamin D activity of lean pork meat appears to be in the range 1.0 to 2.9 μg/170 g meat (average serving size) based on the present data and that of Burild *et al*. (2016). The total vitamin D activity of less lean pork products could be even higher, as Burild *et al*. (2016) have

shown that the total vitamin D activity of fat from animals fed HyD® or vitamin D_3 (2000 IU/kg feed) was 24.0 and 17.7 μg/kg, respectively.

Sensory data in this study show that there was no significant difference in sensory descriptors of the pork loin from any of the treatment groups. This highlights that the addition of vitamin D_3 and HyD® to the diets of pigs did not alter the consumer acceptability of the resulting pork loin from the treatment groups.

In terms of the evidence-base for vitamin D-biofortified beef, again there have been a number of beef heifer studies (Swanek *et al*., 1999; Foote *et al*., 2004; Montgomery *et al*., 2004; Sell *et al* 2004) but which included vitamin D_3 in the finisher feed or as a single oral bolus dose at supra-nutritional levels, that exceed the current EU allowable level of 4000 IU/kg feed (EFSA, 2009). In addition, as the focus of these studies was primarily on the effect of the additional vitamin D on tenderness of beef cuts, only two reported data on the vitamin D and 25-hydroxyvitamin D content of the resulting beef, both of which increased with increasing feed vitamin D content but well above allowable levels at up to 5 million IU vitamin D (Foote *et al*., 2004, Montgomery *et al*., 2004). While a number of other studies have tested the impact of HyD® added to the feed, again with the aim of improving meat quality/tenderness (Wertz *et al*., 2004; Rivera *et al*., 2005; Cho *et al*., 2006; Lawrence *et al*., 2006; Carnagey *et al*., 2008a; 2008b), HyD® is not currently permitted for use in feed of beef heifers in the EU (EFSA, 2009, 2012). It is for these reasons, we tested the effect of vitamin D_3 at the EU maximum allowable level of inclusion, and half that, versus no additional vitamin D_3 added to the finisher diet of beef heifers for 4 weeks. Not unsurprisingly, vitamin D_3 content of the beef steak increased in a dose-related manner with increasing feed level. Interestingly, pooled sample data indicated that the 25-hydroxyvitamin D content of the beef steak appeared to increase about 2-fold when the feed content of vitamin D_3 was increased from 0 to 2000 IU/kg, but then with no further increment at 4000 IU/kg feed, whereas individual sample data showed there was a further significant increase in the 25 hydroxyvitamin D content of the beef from heifers fed the 4000 IU/kg diet in comparison to the 2000 IU/kg data, highlighting the potential requirement for more extensive analysis of food samples in terms of vitamin D food analytics due to the presence of individual differences. This was also the trend for total vitamin D activity. The present data suggests that the total vitamin D activity of beef (rib eye) steak appears to be around 1.5 μg/144 g meat (average serving size).

The present sensory data suggest that there was no significant difference in the overall acceptability of the beef steak from heifers that received the 2000 IU vitamin D_3/kg diet or

4000 IU vitamin D_3/kg treatment diets. However, 0 IU vitamin D_3/kg treatment group had significantly lower overall acceptability scores compared to both vitamin D treatments groups. A 0 IU vitamin D_3/kg treatment group was included in this study as a reference group, but in practise it is unlikely there would be zero addition to finishing feed. Thus, the addition of vitamin D_3 to the diets of beef heifers did not negatively impact the consumer acceptability of the resulting beef steak from the 2000 IU and 4000 IU vitamin D_3/kg dietary treatment groups.

Public health strategies to date have largely focused on supplementation as a means to prevent vitamin D deficiency; however, this only works for those who consume vitamin D supplements, of which consumption can be inconsistent across sex, age group, and area of residence (Calvo *et al*., 2005). The most recent NHANES data brief showed that supplemental vitamin D usage increases with increasing age; 26.5% and 33.8%, 38% and 45%, and 44% and 56.3% in males and females, respectively, in the age groups, 20-39 years, 40-59 years, and >60 years, respectively (Gahche *et al*., 2011). Importantly, the most recent data for vitamin D status in the US population indicated that 24% of individuals over 1 year have a serum 25(OH)D <50 nmol/L (Schleicher *et al*, 2016). So, while the report indicated that supplement use in the population was common, there is still a high proportion of the population who do not take a vitamin D supplement and in addition, approximately one third of the population with a low vitamin D status. Thus, it is important to consider further options in addition to supplementation policies when aiming to improve vitamin D status in the population. Similarly, mandatory fortification of one staple food does address those which are non-consumers or low consumers of that food (Cashman & Kiely, 2011). Hence, it becomes important to consider the diversity that exists in dietary patterns and careful consideration must be placed on the choice of foods to fortify in order to best meet the needs of the population and also to ensure safety (Cashman, 2015). The present work shows that small to moderate increases can be made to the total vitamin D activity of eggs, pork and beef, following the addition of vitamin D_3 and/or HyD® to animal feedstuffs, where permissible, and at levels that adhere to current EU regulations. This work adds to the evidence-base that vitamin D biofortified eggs and meats can be produced with enhanced vitamin D content and also maintain consumer acceptability. Evidence of the effectiveness of vitamin D biofortified foods in terms of preventing vitamin D deficiency is a clear priority to bring their concept closer to more widespread availability and use.

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Chapter 5

Effect of vitamin D bio-fortified eggs on wintertime serum 25-hydroxyvitamin D concentrations in adults: Randomised controlled trial.

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5.1 Introduction

Vitamin D intakes are lower than dietary recommendations in most populations and increasing evidence suggests that the current dietary supply is unable to offset the lack of cutaneous production of vitamin D throughout the winter months in northerly latitudes (Kiely & Black, 2012). Recent data suggest that one in eight Europeans are vitamin D deficient (Cashman *et al*, 2016), thus strategies to improve the vitamin D status of the general population are urgently required. The fortification of food with vitamin D has been suggested as a strategy with potentially widest reach and impact in the population in terms of enhancing vitamin D intakes and minimizing risk of vitamin D deficiency.

Traditional fortification practices in which exogenous vitamin D is added to foods has been and will continue to be an important approach for increasing the content of vitamin D. For example, Black *et al.* (2012) in their systematic review and meta-analysis of vitamin D fortified foods and vitamin D status in randomised controlled trials (RCTs), highlighted the efficacy of vitamin D food fortification in increasing serum 25-hydroxyvitamin D [25(OH)D] concentrations in a dose-dependent manner. However, the authors also suggested that the RCTs included were not primarily designed to assess the impact of food fortified with vitamin D on serum 25(OH)D concentrations or prevention of vitamin D deficiency. It has also been suggested that food fortification strategies should account for diversity in the diet (Cashman & Kiely, 2015). Thus, the use of a 'bio-fortification' (also referred to as 'bio-addition' [Calvo & Whiting, 2013]) approach to enhance the vitamin D content of foods also merits serious attention not only as it may hold more consumer appeal in some cases, but also as it may increase other metabolites of vitamin D which would boost the overall relative effectiveness of these foods in raising vitamin D status (Cashman, 2015). For example, as outlined in Chapter 4, there have been several reports illustrating that the vitamin D_3 content of eggs can be significantly increased by the greater addition of vitamin D_3 to the feed of hens (Mattila *et al*, 1999; 2003; 2004; 2011; Yao *et al*, 2013; Browning & Cowieson, 2014 and Cashman *et al*, 2015), albeit several of the studies (Mattila *et al*, 2003; 2004; Yao *et al*, 2013 and Browning & Cowieson, 2014) use levels of inclusion above the upper allowable level for feeds in Europe [3000 IU/kg diet; European Union Register of Feed Additives pursuant to Regulation (EC) 1831/2003)]. Addition of commercially available 25-hydroxyvitamin D_3 (HyD®) to the diet of hens has also been shown to increase egg 25-hydroxyvitamin D_3 content (Mattila *et al*, 2011; Browning & Cowieson, 2014; Cashman *et al*, 2015), albeit two studies used HyD® at some levels above the upper allowable level [0.080 mg/kg diet (EFSA, 2005)].

There are however several additional factors to acknowledge when examining the effectiveness of bio-fortified foods as opposed to fortified food and supplements, such as the food matrix where the vitamin D compounds are bound and any impact this may have on their bioavailability once consumed, and also any effect that processing or cooking may have on the retention of vitamin D and 25-hydroxyvitamin D in these foods before consumption (Jakobsen *et al*, 2014; Taylor *et al*, 2014). In order to provide proof of efficacy of these foods and to address these considerations, such foods should be examined in a specifically designed food-based intervention trial. Therefore, the objective of the present work was to investigate the effect of consumption of vitamin D_3- or HyD® -biofortified eggs on winter serum 25(OH)D concentrations in adults using an 8-week RCT.

5.2 Subjects and Methods

5.2.1 Subjects for RCT

A total of 55 apparently healthy, free-living adults aged 45-70 years were recruited to this eight-week food-based vitamin D intervention trial. Subjects were recruited in the Cork area through the use of advertisements placed around University College Cork and across the location. We aimed to recruit approximately equal numbers of men and women. Inclusion criteria were consenting white men and women aged 45-70 y. Volunteers were excluded if they were unwilling to discontinue consumption of vitamin D-containing supplements for four weeks before initiation of the study and throughout the study. Volunteers were also excluded if they planned to take a winter vacation (during the course of the eight-week intervention) to a location at which either the altitude or the latitude would be predicted to result in significant cutaneous vitamin D synthesis from solar radiation (e.g. a winter-sun coastal resort or a mountain ski resort) or if they used tanning facilities of any type. A severe medical illness, allergy to egg products, medically advised to limit egg intake in relation to managing hypercholesterolemia, hypercalcaemia, known intestinal malabsorption syndrome, excessive alcohol use, the use of medications known to interfere with vitamin D metabolism also were reasons for exclusion. The study was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals, University College Cork. All participants gave their written informed consent according to the Helsinki Declaration. The study was registered on clinicaltrials.gov at NCT02678364.

5.2.2 Rationale and design of RCT

The study was a controlled, food-based intervention trial in which adults (aged 45-70 y) were randomized to three groups: a control group who habitually consumed only two eggs or less per week and who agreed to do so for the duration of the RCT; and two vitamin D groups, who were habitual egg consumers and willing to consume seven eggs per week, who received either seven vitamin D_3-enhanced eggs (Vitamin D_3-eggs group) or seven HyD®-enhanced eggs (HyD® eggs group) a week for eight weeks. The rationale for the inclusion of a control group was to show that serum 25(OH)D concentration decreased over the eight weeks of the winter RCT, akin to a placebo group in our previous vitamin D RCTs (Cashman *et al*, 2008; 2009; 2011). Our initial hen feeding trial showed that even using only half the upper allowable level of vitamin D in feeds led to eggs with considerable total vitamin D activity (2.8 μg/egg) (Cashman *et al*, 2015), thus, it wasn't feasible to supply seven eggs a week and not impact on vitamin D status of the control group. Therefore, we chose to limit the egg consumption to a maximum of two per week (and participants were not required to consume these two eggs but rather if they did consume eggs to limit them to two per week), and in terms of feasibility we selected habitual low consumers of eggs for this control group to enhance compliance over eight weeks. We felt this was a more feasible approach than requesting participants who were habitual egg consumers but then stratified to the control group to limit their egg intake to two maximum over eight weeks, which could be challenging. The two vitamin D groups were egg consumers willing to consume seven eggs per week. Seven and 1-2 eggs a week (including eggs in dishes) is consistent with that consumed in the highest and lowest tertile of adult egg consumers in Ireland, respectively, based on data from the National Adult Nutrition Survey (NANS, 2011). From a dietary guideline perspective, the general population can include up to seven eggs a week in their diet (Eckel *et al*, 2013). The random assignment of subjects in the two vitamin D-enhanced eggs groups was centralised and computer-generated, and accounted for sex.

The vitamin D intake from food sources in the control group would be expected to be close to that in general adult population aged 50-70 y (median ~5 μg/d; Black *et al*, 2012), whereas the intakes in the two vitamin D-enhanced eggs groups were expected to be ~10 μg/d, based on the additional vitamin D activity provided by these eggs. The ~10 μg/d in HyD®-eggs group, however, is based on an assumption that the 25-hydroxyvitamin D in the eggs will behave in a similar manner to synthetic 25-hydroxyvitamin D used by us in our previous RCT of older adults and which was seen to increase serum 25(OH)D more effectively (1 μg/d 25-hydroxyvitamin D consumed equivalent to 5 μg/d of vitamin D_3

consumed; Cashman *et al*, 2012), and for which some Food Composition tables assume it does (FSA, 2008; Saxholt *et al*, 2008; Zurich, 2010). However, as mentioned above, it may be that the bioavailability of 25-hydroxyvitamin D means that at worst it will only be of equivalence to vitamin D_3, as suggested by some researchers (Jakobsen *et al*, 2007). Thus, intakes of vitamin D may be only 6.5 μg/d in the HyD®-eggs group.

5.2.3 Eggs for RCT

Both forms of vitamin D are approved by the European Commission expert group (Panel on Additives and Products or Substances used in Animal Feed) for inclusion in animal feedstuffs [European Union Register of Feed Additives pursuant to Regulation (EC) 1831/2003; EFSA, 2005], and the vitamin D_3/HyD®-enhanced eggs for the RCT were supplied on a weekly basis by the School of Agricultural and Food Science, University College Dublin. The experimental vitamin D_3- and HyD®-containing hen diets, each produced at the beginning of the feeding trial in 100 kg batches, were based on a basic diet containing crude protein, 155 g/kg, and metabolisable energy, 10.44 MJ/kg. The diets were also balanced for amino acid profiles and fatty acid content, and were provided in a layer mash form. Information on the composition of the hen diet used in production of commercially available eggs was not known to us.

The eggs were placed in specifically purchased cardboard egg boxes, labelled with 'use by date', and the subject IDs, all by staff not involved in the conduct of the RCT. The control (non-vitamin D-enhanced) eggs were purchased locally at a major retail store and were provided to all in the control group for consistency. The vitamin D_3 and 25-hydroxyvitamin D_3 contents of the three types of eggs used in this trial (i.e., commercially available [control] eggs, the vitamin D_3-enhanced eggs and the HyD®-enhanced eggs) were assessed by in-house HPLC analysis within the *Cork Centre for Vitamin D and Nutrition Research* (CCVDNR), University College Cork.

5.2.4 Conduct of RCT

The study was carried out in Cork, Republic of Ireland (latitude: 51ºN). All subjects were screened, recruited, and commenced the intervention study between the 5th to 30th January 2015, and finished the study eight weeks later between 3rd and 30th March, 2015, during which the vitamin D status would be expected to decline to a nadir (Webb *et al*, 1988). During the intervention phase, researchers met each participant on two sampling occasions at the human dietary studies unit at the CCVDNR, once each at baseline (week 0) and the endpoint (week 8) of the study. At each visit, an overnight fasting blood sample

was taken from each participant between 08.30 and 10.30 hours by a clinical research nurse. Blood was collected by venepuncture into an evacuated tube with no additive and processed to serum, which was immediately stored at -80ºC until required for analysis. Anthropometric measures, including height and weight, were taken as described previously (Lucey *et al*, 2008). Habitual intake of vitamin D (and calcium) was estimated via a validated food-frequency questionnaire (FFQ) (Collins, 2006; Kiely *et al*, 2016) that was administered by a research nutritionist at baseline. This FFQ, which has been tailored using national food consumption survey data obtained from Irish adults (aged 18-64 y) so as to identify foods that contribute 95% of vitamin D intake, has been shown to have a generally good level of agreement with a 14 day diet history method, and without significant levels of overall bias or proportional bias (Kiely *et al*, 2016). It is important to note that the FFQ was not designed to measure energy or other nutrient intakes and is designed to be administered by a trained researcher in association with validated methods of estimating portion size (Kiely *et al*, 2016). In addition, a health and lifestyle questionnaire, which assessed physical activity, general health, smoking status and alcohol consumption, was completed at baseline.

Participants either collected their weekly/fortnightly allocation of fresh eggs at the human dietary studies unit or in some cases where this was not feasible, the eggs were delivered to them. This regular contact also served to promote compliance and encourage completion of the study protocol. The participants were asked to use the study provided eggs in place of their normally purchased eggs for the duration of the study period. Subjects were requested to use the whole egg, in light of the fact that egg white is low in vitamin D content (Fraser & Emtage, 1976). Subjects were provided with cooking suggestions and meal ideas for incorporation of their vitamin D-enhanced whole eggs into their weekly diet. Compliance was assessed by an egg diary. While the control group were aware of their allocation by virtue of being limited to 2 eggs per week, the allocation of the two vitamin D-enhanced egg groups remained concealed from both participants and staff involved in the conduct of the RCT until the final analyses was complete. The biochemical outcome measures were reported by people who were masked to all subjects' allocation scheme.

5.2.5. Laboratory analysis

High performance liquid chromatography analysis of RCT eggs.

The separated yolks from six individual eggs per treatment were analysed for vitamin D_3 and 25-hydroxyvitamin D by using an in-house high performance liquid chromatography (HPLC) method at the CCVDNR. The extraction procedure was adapted from Jakobsen *et al*. (2004), and following semi-preparative steps also described elsewhere (Jakobsen *et al*, 2004; 2007), vitamin D_3 and 25-hydroxyvitamin D were quantified using a HPLC system (Shimadzu Corporation, Kyoto, Japan) consisting of two LC20ADXR pumps, SIL-30AC autosampler, CTO-20AC column oven, SPD-30MA PDA detector, FRC-10A fraction collector and a CBM-20A system controller. Concentrations of vitamin D_3 and 25-hydroxyvitamin D_3 were calculated based on external calibration and adjusted for percent recovery of the internal standard. In addition, as the eggs from our original hen feeding trial were analysed by a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method at the National Food Institute, Technical University of Denmark (Burild *et al*, 2014), the data were aligned with that method to ensure comparability. The National Food Institute laboratory has been accredited for vitamin D and 25-hydroxyvitamin D since 1994 and 2004, respectively. For the last 15 years, it has performed the analysis for vitamin D and 25-hydroxyvitamin D in food for the Danish and Norwegian Food Composition Tables.

Serum 25-hydroxyvitamin D.

Concentrations of total 25(OH)D [i.e., 25(OH)D_2 plus 25(OH)D_3] in all serum samples were measured by the CCVDNR by using a LC-MS/MS method that has been described in detail elsewhere (Cashman *et al*, 2103). The intra-assay CV of the method was <5% for all 25-hydroxyvitamin D metabolites, while the inter-assay CV was <6%. The CCVDNR is a participant in the Vitamin D Standardization Program (Sempos *et al*, 2012) and is certified by the Centers for Disease Control and Prevention's Vitamin D Standardization Certification Program (Rahmani *et al*, 2013). In addition, the quality and accuracy of the serum 25(OH)D analysis by using the LC-MS/MS in our laboratory is monitored on an on-going basis by participation in the Vitamin D External Quality Assessment Scheme (Charing Cross Hospital, London, UK).

Serum intact parathyroid hormone.

Serum PTH concentrations were measured at CCVDNR in all serum samples by using an ELISA [Intact parathyroid hormone, MD Biosciences Inc., St. Paul, MN 55108]. The intra- and inter-assay CVs were 3.4% and 3.8%, respectively.

Serum total calcium and cholesterol.

Total calcium, albumin and cholesterol concentrations were measured at the CCVDNR in all serum samples by using a fully automated clinical analyser (RX Monaco Randox Laboratories Ltd., Co. Antrim, UK). The inter-assay CVs were 2.2%, 1.9% and 2.2%, for total calcium, albumin and cholesterol, respectively. Serum calcium concentrations were adjusted for albumin concentrations.

5.2.6 Sample size estimation

We wished to be able to compare the endpoint (March) serum 25(OH)D concentration between the control group and that in both of the vitamin D-enhanced egg groups, which were predicted to have ~5 μg/d additional vitamin D. Our previous vitamin D RCTs in young and older adults (Cashman *et al*, 2008; 2009) have shown that there was a 12 nmol/L, on average, higher endpoint serum 25(OH)D concentration in the 5 μg/d vitamin D treatment groups compared to respective placebo groups. On the basis of the distribution of serum 25(OH)D data from our previous study of older adults which was at the same time of year (Cashman *et al*, 2012), our power estimates based on a 12 nmol/L difference in serum 25(OH)D between the control group and either of the vitamin D-enhanced eggs groups indicated a need for 17 volunteers/group (*n* 51 in total) for 90% assurance at α=0.05. We did not power the study to detect differences between the two vitamin D-enhanced egg groups, as this was not the primary objective.

5.2.7 Statistical analysis

Statistical analysis of the RCT data was conducted using SPSS© for Windows™ Version 20.0 (SPSS, Inc., Chicago, IL, USA). The distributions of all variables were tested with Kolmogorov-Smirnov tests. Descriptive statistics (means ± SD or medians and interquartile range [IQR]; when appropriate) were determined for all variables. One-factor ANOVA was used to compare mean total vitamin D activity of three types of RCT eggs. Dietary calcium and serum PTH were not normally distributed and thus were log transformed to achieve near-normal distributions. Serum concentrations of 25(OH)D, albumin-corrected calcium, and total cholesterol as well as age, weight, height, BMI and dietary vitamin D were normally distributed. Baseline characteristics of male and female

subjects were compared using unpaired Student's *t* tests. Baseline characteristics of subjects in the different intervention groups were compared using Chi-square (for ratio of men to women) and one-factor ANOVA. Analysis of covariance (ANCOVA) (with adjustment for baseline parameter being tested) was used to test between intervention group effects of treatment on weight as well as serum 25(OH)D, PTH, albumin-corrected calcium, and total cholesterol concentrations, and the Tukey's test was used for *post hoc* analysis. Linear models of the response in a repeated measures analysis for the differences in weight as well as serum 25(OH)D, PTH, albumin-corrected calcium, and total cholesterol concentrations were also constructed. Main effects included dietary treatment and sex. Linear models also included two-way interactions between the main effects. Paired *t*-tests were used to test within intervention group changes from pre- to post-intervention concentrations of serum 25(OH)D, PTH, albumin-corrected calcium, and total cholesterol concentrations. Subjects included in the analyses and those not included (i.e., dropouts) were compared on selected characteristics with two sample *t*-tests (continuous variables) and χ^2-tests (categorical variables). A *P*-value of <0.05 was taken as being statistically significant.

An intention to treat analysis of the serum 25(OH)D data as the primary outcome measure was also performed to account for the 4 subjects who dropped out. The 4 missing post-intervention values for serum 25(OH)D were imputed using the multiple imputation function in SPSS ('k' = 5), as suggested by Armijo-Olivo *et al*. (2009). This analysis was viewed as confirmatory of that in which the 4 dropouts were excluded.

5.3 Results

5.3.1 Vitamin D activity of eggs for RCT

HPLC analysis of the three types of eggs used in the present RCT (*n* 6 eggs/group) showed that the mean ± SD vitamin D_3; 25-hydroxyvitamin D_3; and total vitamin D activity (i.e., vitamin D_3 + [25-hydroxyvitamin D_3 × 5]) for vitamin D_3-eggs were 1.04 ± 0.54, 0.50 ± 0.10, and 3.54 ± 1.04 μg/egg, respectively. The mean ± SD vitamin D_3; 25-hydroxyvitamin D_3; and total vitamin D activity for HyD® eggs were 0.14 ± 0.08, 0.88 ± 0.26, and 4.54 ± 1.38 μg/egg, respectively. The mean ± SD vitamin D_3; 25-hydroxyvitamin D_3; and total vitamin D activity for the commercially available (control) eggs were 0.63 ± 0.22, 0.56 ± 0.22, and 3.43 ± 1.31 μg/egg, respectively. There was no significant difference in mean total vitamin D activity across the three types of eggs (P=0.13; ANOVA).

5.3.2. Baseline characteristics of subjects in the RCT

Of 55 subjects recruited onto the study, 51 subjects completed the intervention. The progress of these subjects through the trial is shown in **Figure 1**. Baseline characteristics of subjects who entered the intervention are shown in **Table 1.** Although women were, on average, lighter and smaller than men (both P<0.0001) and had lower BMI (P<0.05) and habitual intake of calcium (P<0.05), there was no difference (P>0.2 for all) in the mean age, habitual intake of vitamin D, serum 25(OH)D, PTH, albumin-corrected calcium or cholesterol concentrations between men and women at baseline (data not shown).

5.3.3 Effects of intervention with vitamin D-enhanced eggs

There was no difference (P>0.3) in mean age, weight, height, or BMI at baseline in the 3 treatment groups (control, Vitamin D_3-eggs and HyD®-eggs groups) (**Table 1** and **2**). Similarly, there was no significant difference (P>0.9) in the proportion of men to women in treatment groups (Table 1). Habitual dietary vitamin D and calcium intakes are shown in Table 1, and these intakes were similar in the 3 treatment groups (P>0.1).

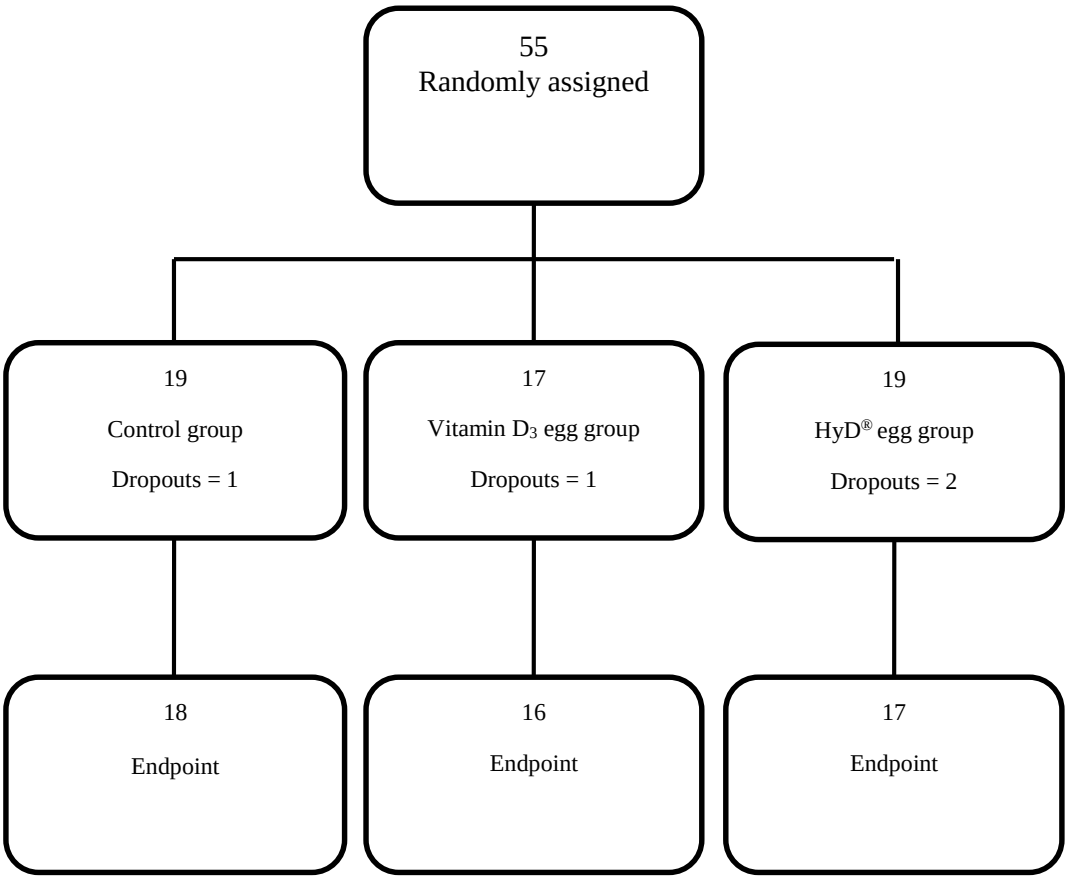

Figure 1. Flow of subjects through the study

Table 1. Selected baseline characteristics of the subjects who entered the intervention study[1].

Treatment groups	Control	Vitamin D_3 eggs	HyD® eggs	*P*-value[2]
n	19	17	19	-
Sex (male: female)	9:10	8:9	10:9	0.930
Age (y)	55.8 (7.5)[3]	56.2 (5.5)	53.8 (5.8)	0.498
Height (m)	1.70 (0.12)	1.70 (0.09)	1.71 (0.09)	0.807
BMI (kg/m^2)	25.8 (3.5)	26.5 (5.0)	24.4 (3.7)	0.300
Habitual dietary vitamin D (μg/d)	6.0 (4.0)	6.7 (3.5)	6.9 (4.0)	0.796
Habitual dietary calcium (mg/d)	842 [670, 1014][4]	961 [764, 1730]	762 [666, 1257]	0.115

[1]BMI,body mass index; PTH, Parathyroid hormone; serum 25(OH)D, serum 25-hydroxyvitamin D; HyD®,supplemental 25-hydroxyvitamin D_3

[2]For baseline comparisons by intervention group: One –way ANOVA

[3] Mean (standard deviation) (all such values)

[4] Median (interquartile range) of non-normally distributed variables (all such values)

Table 2. Weight, serum total cholesterol, 25(OH)D and PTH concentrations in treatment groups at baseline and endpoint of the 8 week intervention study in apparently healthy adults[1].

Treatment groups	Control	Vitamin D_3 eggs	HyD® eggs	*P*-value[2]
n (baseline, endpoint)	(19,18)	(17,16)	(19,17)	-
Weight (kg)[3]:				
pre-intervention[6]	75.1 (12.7)[4]	78.3 (20.1)	73.4 (16.1)	0.654
post-intervention[7]	72.8 (15.6)	78.2 (19.9)	72.3 915.6)	0.387
Serum total cholesterol (mmol/L)[3]:				
pre-intervention[6]	5.2 (0.8)	5.3 (0.9)	5.5 (0.8)	0.435
post-intervention[7]	5.0 (0.8)	5.2 (0.9)	5.4 (0.6)	0.463
Serum 25(OH)D (nmol/L)[3]:				
pre-intervention[6]	41.2 (14.1)	48.2 (18.9)	49.4 (15.8)	0.102
post-intervention[7]	34.8 (11.4)[a*]	50.4(21.4)[b]	49.2(16.5)[b]	<0.0001
Serum PTH (pg/ml)[3]:				
pre-intervention[6]	41.5 [35.0, 62.3][5]	48.9 [40.4, 53.9]	43.0 [39.6, 59.5]	0.991
post-intervention[7]	47.0 [39.0, 68.4]	47.7 [36.8, 58.5]	44.9 [37.4, 58.6]	0.606

[1]BMI,body mass index; PTH, Parathyroid hormone; serum 25(OH)D, serum 25-hydroxyvitamin D; HyD®,supplemental 25-hydroxyvitamin D_3

[2] For endpoint comparison by intervention group: ANCOVA (with adjustment for baseline parameter being tested) followed by Tukeys; [a,b] Different superscript letters represent significant ($P \leq 0.005$) differences among group means

[3] Repeated measures ANOVA was also used to test the treatment × time interaction and the same trend was observed for weight (P= 0.38), serum total cholesterol (P=0.86), serum 25(OH)D (P = 0.0001), and PTH (P=0.6). There was no significant (P> 0.2)in all cases) interaction with sex.

[4] Mean (standard deviation) (all such values)

[5] Median (interquartile range) of non-normally distributed variables (all such values)

[6] All baseline bloods were taken between 5th to 30th of January, 2015.

[7] All endpoint bloods were taken between 3rd to 30th of March, 2015.

[*] Values significantly different from baseline value within a group; paired t-test; P = 0.001

There were no adverse events reported during the study. Of the four dropouts, one subject was from the control group, one subject from the Vitamin D_3-eggs group, and two subjects from the HyD®-eggs group. Dropout during the intervention phase was for reasons of loss of interest or a back injury, and in no instance was dropout related to the intervention. The 51 subjects who completed the study did not differ from the four subjects who dropped out with respect to age, sex, weight, height, BMI, vitamin D intake, calcium intake, or baseline serum 25(OH)D, PTH, serum albumin-adjusted serum calcium or total cholesterol concentrations (P>0.16).

There was good adherence with egg consumption protocols on the basis of the egg diary entries (median [IQR] compliance was 98.2% [93.7; 100%], and compliance was similar among the treatment groups; P=0.6).

Data on egg compliance and measured vitamin D and 25-hydroxyvitamin D contents of RCT eggs, as well as habitual vitamin D intake data from the FFQ were used to estimate daily total vitamin D intakes throughout the study period. Using the factor of 5, the mean ± SD total vitamin D intake was 9.5 ± 3.5, 10.4 ± 4.0, and 6.6 ± 3.9 µg/d in the Vitamin D_3-eggs, HyD®-eggs, and control group, respectively.

There was no difference in mean serum total cholesterol or body weight at baseline (P>0.4) and no significant change in weight (P=0.39) or serum total cholesterol (P=0.46) from pre- to post-intervention in the three treatment groups (Table 2).

Effects of vitamin D egg treatment on serum 25(OH)D and PTH at the baseline and endpoint are shown in Table 2. While the pre-intervention serum 25(OH)D concentration of the control group was lower than that of the other two groups, it was not significantly so (Table 2). Repeated measures ANOVA showed a significant treatment × time effect (P=0.0001). Within group analysis showed that while the control group had a significant decrease in serum 25(OH)D over the 8 week winter intervention period (mean ± SD: 6.4 ± 6.7 nmol/L; P=0.001), there was no significant change in serum 25(OH)D over the same eight week period in the Vitamin D_3-eggs and HyD®-eggs groups (P>0.1 for both). ANCOVA also showed there was a significant (P=0.001) effect of treatment on post-intervention serum 25(OH)D concentrations, whereby adjusting for pre-intervention serum 25(OH)D concentrations, the Vitamin D_3-eggs group and HyD®-eggs group had significantly higher (P≤0.005) post-intervention serum 25(OH)D concentrations relative to that in the control group. There was no significant (P>0.3) difference in post-intervention serum 25(OH)D concentrations between the Vitamin D_3-eggs and HyD®-eggs groups (Table 2).

The percentage (*n*/total group *n*) of subjects in each of the three groups that had post-intervention serum 25(OH)D concentrations <25 nmol/L (the UK and EU threshold of vitamin D deficiency; SACN, 2016; Scientific Committee for Food (SCF), 1993) were 22.2% (4/18), 0% (0/16) and 0% (0/17) for control, Vitamin D_3-eggs and HyD®-eggs groups, respectively (P=0.019). The percentage (*n*) of subjects in each of the three groups that had post-intervention serum 25(OH)D concentrations <30 nmol/L (the US threshold of vitamin D deficiency; IOM, 2011) were 38.9% (7/18), 18.8% (3/16) and 5.9% (1/17) for control, Vitamin D_3-eggs and HyD®-eggs groups, respectively (P=0.057).

There was no significant difference in pre- or post-intervention serum PTH concentrations in the 3 treatment groups (Table 1). Repeated measures ANOVA confirmed a lack of significant treatment × time effect (P>0.6). Within group analysis showed that while there was a trend towards a significant increase in serum PTH (P=0.050) in the control group over the eight week winter intervention period, there was no significant change in serum PTH over the same eight week period in the Vitamin D_3-eggs and HyD®-eggs groups (P>0.7 for both).

There was no significant interaction between treatment and sex in any of the serum 25(OH)D or PTH statistical analyses (P>0.18 for both).

An intention to treat analysis of post-intervention serum 25(OH)D (using imputted values for the 4 subjects who dropped out during the study) showed similar findings ($P\leq$0.0001), and again with a significant decrease in the control group ($P\leq$0.002) but no significant difference in either of the two vitamin D-enhanced eggs treatment groups (P>0.17) (data not shown).

5.4 Discussion

Despite numerous vitamin D and/or HyD® hen feeding studies over the last 15 years, which collectively have clearly demonstrated the viability of increasing the vitamin D and/or 25-hydroxyvitamin D content of eggs (Mattila *et al*, 1999; 2003; 2004; 2011; Yao *et al*, 2013; Browning & Cowieson, 2014; Cashman *et al*, 2015; Chapter 4), the present study, to our knowledge, is the first to investigate the effect of such vitamin D-biofortified eggs on vitamin D status in humans. The present RCT showed that consumption of seven vitamin D- or HyD®-enhanced eggs per week, which were shown to possess consumer acceptability (Chapter 4), maintained serum 25(OH)D concentrations in healthy adults and protected against its decline during the 8 weeks of winter. Such a decline in serum 25(OH)D concentrations between January and March was evident in the control group, which consumed no more than 2 commercially available eggs per week. This decline in serum 25(OH)D is in line with that reported in the placebo groups in our previous RCTs of adults over a similar time-frame during winter (Cashman *et al*, 2012; 2014). Also of note, the vitamin D-biofortified eggs, which exhibited these vitamin D status protective effect, were achieved by addition of either vitamin D_3 (3000 IU/kg diet) or HyD® (0.075 mg/kg diet; equivalent to 3000 IU/kg diet) to the hens' feeds at levels allowable by European Council directives (European Union Register of Feed Additives pursuant to Regulation (EC) 1831/2003; EFSA, 2005), whereas the majority of previous studies used levels exceeding these maximum allowable contents (Mattila *et al*, 1999; 2003; 2004; Yao *et al*, 2013; Browning & Cowieson, 2014).

The IOM in 2011 set an EAR for vitamin D of 10 μg/day; this was meant to ensure a serum 25(OH)D concentration above 40 nmol/L in at least half the population when potential for UVB-induced dermal synthesis was absent or minimal (i.e., in extended winter). The findings of the present study, which was carried out in the winter months in Cork, Ireland (51°N), are consistent with this in that consumption of the vitamin D enhanced eggs as part of the participants' usual diet, which led to a mean daily intake (MDI) of vitamin D of ~10 μg/d, resulted in post-intervention mean serum 25(OH)D concentrations >40 nmol/L. The vitamin D MDI of ~10 μg/d in both vitamin D enhanced-egg groups was also sufficient to prevent, or dramatically diminish, the prevalence of vitamin D deficiency, depending on the selection of serum 25(OH)D threshold to define same. For example, none of the participants in either of the two vitamin D enhanced-egg groups had post-intervention serum 25(OH)D <25 nmol/L [the UK and EU definition of vitamin D deficiency (SCF, 1993; SACN, 2016)], whereas 6-19% of participants had post-intervention serum 25(OH)D <30 nmol/L [the US definition of vitamin D deficiency (IOM, 2011)]. In contrast, the prevalence of serum 25(OH)D concentration <25 and <30 nmol/L was 22% and 39%, respectively, in the control group.

However, as baseline serum 25(OH)D concentrations were lower, even if not significantly so, in this group compared to the two vitamin D-enhanced eggs groups, it may have led to the slightly higher endpoint prevalence of vitamin D deficiency compared to that in placebo groups in our previous vitamin D RCTs (~33%, on average, with serum 25(OH)D <30 nmol/L; Cashman *et al*, 2008; 2009; 2014).

The use of 25-hydroxyvitamin D, available as HyD®, in animal nutrition allows for an increase in the 25-hydroxyvitamin D content in foods of animal origin. It remains to be established if the food matrix affects the higher potency of 25-hydroxyvitamin D as discussed earlier. The present study did not set out specifically to answer the question of whether the relative potency of 25-hydroxyvitamin D to vitamin D_3 is 5 or some other factor, as assessed in previous human and pig studies (Rossini *et* al, 2005; Jakobsen *et al*, 2007; Cashman *et al*, 2012; Jetter *et al*, 2014; Burild *et al*, 2016), rather it wished to test the impact of vitamin D-enhanced eggs on human vitamin D status. That 25-hydroxyvitamin D has a higher effectiveness in terms of raising serum 25(OH)D than vitamin D_3 is assumed within our study. For example, calculation of total vitamin D activity of the eggs applies a conversion factor of 5 for 25-hydroxyvitamin D, as reported by us previously (Cashman *et al*, 2012) and which is used in several Food Composition tables (FSA, 2008; Zurich, 2010; Saxholt *et al*, 2008). Using these total vitamin D activity estimates meant that the Vitamin D_3-eggs and HyD®-eggs groups received an additional 3.5 and 4.5 μg/d of vitamin D, respectively, which together with the habitual dietary vitamin D yielded a MDI of ~10 μg/d. Of note, the current USDA National Nutrient Database for Standard Reference suggests the vitamin D content of 100 g raw egg is 2 μg, an estimate that does not include 25-hydroxyvitamin D. Accordingly, vitamin D intake estimates from NHANES in the US do not account for the contribution of 25-hydroxyvitamin D (Fulgoni *et al*, 2011). Taylor *et al*. (2014) recently performed some modelling to include food-derived 25-hydroxyvitamin D in intake estimates for US adults, an effect which would be even more pronounced should vitamin D-enhanced eggs become more widespread in the US. For example, the total vitamin D content of the eggs used in the analysis by Taylor *et al*. (2014) at 5.7 μg/100 g whole egg, and which was very similar to that in the commercial eggs and vitamin D_3-enhanced eggs in this study (5.7 and 6.0 μg/100 g whole egg, respectively), could be increased to 7.7 μg/100 g whole egg if HyD®-eggs were used. In the US, the FDA designated 25-hydroxyvitamin D as having GRAS (generally recognised as safe) status for inclusion in chicken feeds (0.069 mg/kg diet) (FDA, 2007).

As there is a persistent public perception that egg consumption is associated with increased risk of coronary heart disease following previous recommendations to limit dietary cholesterol

(Krauss *et al*. 2000), serum total cholesterol was measured as a secondary outcome in the current study. Consumption of 7 eggs a week as part of the current intervention had no effect on serum total cholesterol. While the 2010 *Dietary Guidelines for Americans* suggested to keep dietary cholesterol intakes less than 300 mg/d, the more recent this version of the guidelines (2015 *Dietary Guidelines for Americans*) has not included this recommendation. Furthermore, recent recommendations have stated that there is insufficient evidence to determine whether reducing dietary cholesterol reduces low-density lipoprotein cholesterol (Eckel *et al.* 2013). The present study did not include a wider dietary assessment tool and thus did not assess the impact of consumption of 7 vitamin D-enhanced eggs a week on fat or energy intake; however, body weight and, as mentioned already, serum cholesterol were unaffected over the 8 weeks of the RCT.

A strength of the present study was that it was carried out in free-living adults, providing a true to life setting. Participants received cooking suggestions and meal ideas for incorporation of their vitamin D-enhanced whole eggs into their weekly diet, and were simply requested to ensure that they used the whole egg in their dishes, in order to consume to the full amount of vitamin D and that only themselves consumed the dishes to prevent dilution of the treatment effect. Cooking methods chosen by the participants appear to have had minimal effect on the retention of vitamin D in the egg as the effect of consumption of the additional vitamin D received from the treatment eggs on serum 25(OH)D concentrations was evident. Mattila *et al*. (1999) has shown that vitamin D_3 and 25-hydroxycholecalciferol retentions in egg yolk were good after cooking. Compliance to the egg intervention was also high (98.2%) which may be attributed to the weekly or fortnightly collection of fresh eggs, which allowed for constant communication with the participants to ensure their satisfaction.

In terms of potential limitations, the purposeful selection of habitual low consumers of eggs ($\leq$2 per week) in the control group, while for the pragmatic reasons of feasibility and enhancing compliance to a limited egg consumption, may have led to some degree of allocation bias. It may have contributed to the lower baseline serum 25(OH)D compared to that in the vitamin D-enhanced egg groups, even though it was not significantly so. This, in turn, may have contributed to a lower post-intervention serum 25(OH)D in the control group, and explain the measured difference of 14.4-15.6 nmol/L between the control and vitamin D enhanced eggs groups compared to the more predicted 12 nmol/L difference. The statistical analysis, however, took baseline serum 25(OH)D concentration into account, and moreover, repeated measures ANOVA and *post hoc* tests showed that there was no change in serum 25(OH)D concentration from pre- to post-intervention in either vitamin D-enhanced egg group, despite a significant decrease in the control group.

In conclusion, weekly consumption of 7 vitamin D-enhanced eggs, which are produced by hens provided with feed containing vitamin D (either as vitamin D_3 or HyD®) at the allowable maximum content, has an important impact on winter vitamin D status in adults. These new data have public health relevance in light of the percentage of the population with vitamin D deficiency in winter, but also as one in two adults are reported to include eggs or egg dishes in their diet on a regular basis (NANS, 2011), vitamin D-biofortified eggs are an important dietary strategy aimed at lowering the risk of vitamin D deficiency.

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Chapter 6

Phylloquinone intakes and food sources, vitamin K status, and associations with a serum marker of bone resorption: data from the recent National Adult Nutrition Survey in Ireland

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6.1 Introduction

While it has been suggested that vitamin K is arguably the least known member of the fat-soluble vitamins (Willems *et al*, 2014), the last decade has seen increasing interest in the potential health effects of vitamin K beyond its classical role in blood coagulation. For example, there is evidence of emerging physiological roles for vitamin K in bone biology, vascular calcification, energy metabolism and inflammation, all of which have been comprehensively reviewed elsewhere (Booth, 2009; Shearer *et al*, 2012; Beulens *et al*, 2013; Willems *et al*, 2014). Many of these roles are underpinned by the biochemical function of vitamin K, namely to serve as a cofactor for the vitamin K-dependent γ-carboxylase (a microsomal enzyme that facilitates the post-translational conversion of glutamyl to γ-carboxyglutamyl [Gla] residues) (Esmon *et al*, 1975). The plasma procoagulant, prothrombin, was the first described Gla residue-containing protein, but presently there are at least 14 more Gla residue-containing proteins, which collectively are referred to as the vitamin K dependent protein (VKDP) family (Willems *et al*, 2014). There is also, however, an emerging viewpoint that the biochemical function of vitamin K may extend beyond that of a cofactor for vitamin K-dependent γ-carboxylase (Booth, 2009).

Vitamin K exists naturally in multiple forms, and while not fully reflecting the complexity of biological differences among the various forms (Booth, 2009), they can be viewed as belonging to either phylloquinone (vitamin K_1), menaquinones [vitamin K_2; and comprised of at least 10 compounds (Shea & Booth, 2016)] or menadione (vitamin K_3). Menadione is a 2-methyl-1,4-napthoquinone ring, which is common to all forms of vitamin K and which fulfils the enzyme cofactor role (Booth, 2009). In terms of dietary sources, phylloquinone is the primary form in Western diets. It is a plant-based form which is concentrated in green leafy vegetables and certain plant oils (Suttie, 2009). The menaquinones (MKs), which vary in the number of repeating isoprenoid units on their side-chain to the core methyl-1,4-napthoquinone ring, have a more restricted distribution in the diet (Beulens *et al*, 2013). While MK7-13 have a bacterial origin, being primarily concentrated in animal meats and plant foods, MK4 is not produced by bacteria but instead is either formed from phylloquinone or menadione, and is concentrated in animal meats and dairy products (Shea & Booth *et al*, 2016).

While the maintenance of plasma prothrombin concentrations is the basis for the current recommended dietary intake value of 1 μg/kg body weight per d in the UK (DOH, 1991) and EU (SCF, 1993), the adequate intake (AI) values for vitamin K, established by the US Institute of Medicine (IOM) in 2001 at 90 μg/d for women and 120 μg/d for men, are based on median

intakes from food, as estimated from NHANES III (1988–1994) (IOM, 2001). There is also speculation that these AI values may not be sufficient to attain complete γ-carboxylation of all VKDPs (Binkley *et al*, 2002; Booth *et al*, 2003). These estimates of dietary requirement for vitamin K (DOH, 1991; EC, 1993; IOM, 2001) do not distinguish between phylloquinone and menaquinone intakes, but due to the fact that it is the form which predominates in food composition databases (Beaulens *et al*, 2013; Shea & Booth, 2016) used to generate dietary intake estimates, they are, by default, based on phylloquinone.

It has been recently suggested that a better understanding of the role of vitamin K in health and disease requires the assessment of vitamin K nutritional status in population studies (Shea & Booth, 2016). Dietary intake of vitamin K is one of the primary determinants of vitamin K status (Booth & Al Rajabi, 2008). While no single biomarker of vitamin K status is a robust measure of vitamin K deficiency or sufficiency (Booth & Al Rajabi, 2008), the degree to which serum osteocalcin (OC; a VKDP produced by osteoblasts during bone formation and the most abundant non-collagenous protein in bone) is undercarboxylated (%ucOC) is considered a sensitive indictor of poor vitamin K status in bone (Gundberg *et al*, 1998). While there are a number of VKDPs in bone, the γ-carboxylation of OC is the primary mechanism underpinning the putative protective influence of vitamin K on bone (Booth, 2009). However, vitamin K has also been shown to modulate certain cytokines involved in bone turnover (Shea *et al*, 2008; Katsuyama *et al*, 2005), which may represent an additional mechanism of action. Over a decade ago, we reported that data from our nationally representative nutrition survey of adults in Ireland, aged 18-64 y, highlighted low habitual intakes of phylloquinone, which we suggested may have had potential implications for bone health (Duggan *et al*, 2004). The adult survey at the time, however, did not include blood sampling in its design and thus we had no measure of vitamin K status or biomarker of bone turnover. In addition, as the survey included only adults aged 18-64 y, we have no representative data on older Irish adults (aged >64 y), a subgroup potentially at higher risk of low phylloquinone intakes (IOM, 2001) and who have higher rates of bone turnover and loss (Cashman & Flynn, 1999).

Therefore, the objectives of the present study, based on the most recent national nutrition survey in Ireland [the *National Adult Nutrition Survey* (NANS; IUNA, 2011)], were to estimate the mean daily intake of phylloquinone, the adequacy of phylloquinone intakes and the contribution made by food groups to phylloquinone intakes in adults aged 18–90 years. We also wished to compare thisnewer information on dietary phylloquinone in a subset of NANS adults aged 18-64 y with those similarly-aged from the previous survey over a decade before (Duggan *et al*, 2004). Data from the national nutrition surveys in the UK suggest phylloquinone intakes by adults have been decreasing over the last two decades, consistent

with a concomitant decline in leafy green vegetable consumption (Thane *et al*, 2006). Finally, as the NANS included blood sampling, we measured serum %ucOC as an index of vitamin K status in bone and explored its associations with phylloquinone intake as well as with serum C-terminal telopeptide of type I collagen (CTx), a marker of bone resorption (Chubb, 2012).

6.2 Materials and methods

6.2.1 The National Adult Nutrition Survey sample

A detailed description of the methodology used in NANS, including the sampling procedure as well as sample recruitment has been reported elsewhere (IUNA, 2011; Cashman *et al*, 2013]. Briefly, the fieldwork phase of NANS was carried out between October 2008 and April 2010, providing a seasonal balance to the data and biological sample collection. To achieve a nationally representative sample of community-dwelling adults aged 18 years and over, a quota sampling approach was adopted using data from the 2006 Census (CSO, 2007). A sample of 1500 free-living adults to represent a population of ~4.2 Million participated in the dietary survey. There were few exclusion criteria, other than pregnancy/lactation and inability to complete the survey due to disability. The study was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals, University College Cork and the Human Ethics Research Committee of University College Dublin. All eligible and willing participants gave their written consent according to the Helsinki declaration.

Analysis of the demographic features in this sample has shown it to be a representative sample of Irish adults with respect to age, gender, social class and geographical location when compared to Census data (CSO, 2007; IUNA, 2011; Cashman *et al*, 2013). While participation in the survey did not require provision of a blood sample as an eligibility criterion, all participants were asked if they were willing to provide a blood sample. Of the total group of respondents, 75% provided a blood sample. The demographic features of the group of participants who provided a blood sample and those in the entire sample have been described elsewhere (IUNA, 2011; Cashman *et al*, 2013).

Information on social class and education level, smoking status, alcohol intake, medication usage (including those which contained nutrients) was also collected (IUNA, 2011; Cashman *et al*, 2013). Blood pressure and anthropometric measures including height, weight, waist and hip circumference as well as measures of body composition were taken in the respondents' homes, as described previously (IUNA, 2011). Blood samples were collected by venepuncture into a vacutainer tube by a qualified nurse at designated centers within the survey area or in

the respondent's home if the respondent could not travel. Bloods were transported to the laboratory for further processing and serum was stored at -80^{o}C until required for analysis.

6.2.1.1 Food consumption data

Food intake in NANS was recorded using a 4-d estimated food diary: participants were asked to provide detailed information on the types and amounts of all foods, beverages, and nutritional supplements consumed over a 4-d recording period. The detailed methodology of the 4-d estimated food diary, which was used to collect food and beverage intake data during the survey, and the methods used to quantify food and drink intake are described in detail elsewhere (IUNA, 2011). As one of the aims of the present work was to investigate if temporal changes had occurred in phylloquinone intakes in Irish adults, intake estimates for the 18-64 y-old subgroup of participants in the NANS were compared with those of the previous nationally representative survey of adults (aged 18-64 y) in Ireland (Northern Ireland and Republic of Ireland) from 1997 to 1999, called the North/South Ireland Food Consumption Survey (NSIFCS) (*n*=1379), and which was performed by the same team and using similar sampling and dietary methodology as for NANS. The NSIFCS used a 7-d estimated food diary, and a detailed description of the sampling procedure, survey design, and methodology for the NSIFCS is available (Kiely *et al*, 2001; Harrington *et al*, 2001). Data on the phylloquinone intake estimates for the participants in NSIFCS were published previously (Duggan *et al*, 2004).

6.2.1.2 Vitamin K compositional data

The procedures to update the vitamin K composition data in NANS were along similar lines as that used in the NSIFCS. In brief, while the version of WISP© software used at the time of the analysis of NSIFCS (because it relied on data from the 5th edition of McCance and Widdowson's *The Composition of Foods* (Holland *et al*, 1993 and supplemental volumes) did not include data on phylloquinone levels (and instead we relied on a database established by Bolton-Smith *et al*. (Bolton-Smith *et al*, 2000a) which contained the phylloquinone content of approximately 2000 foods), the more recent version of WISP© software used for the present analysis of NANS food consumption data used the 6th edition of McCance & Widdowson's *The Composition of Foods* (FSA, 2002) and supplement editions as the core food composition databank. In cases where phylloquinone values for foods were not contained in the 6th edition, these were derived by recipe calculation or based on similarity to other foods, as described in detail elsewhere (Duggan *et al*, 2002). In some instances the data from the database established for the NSIFCS was used to augment the 6th edition of *The Composition of Foods* database, and phylloquinone levels from the USDA National

Nutrient Database for Standard Reference (US Department of Agriculture, 2016) were used in a few food codes.

Nutritional supplement intake data were collected from the prospective food diary because participants were requested to record nutritional supplements as consumed and these data were confirmed in a face-to-face interview, as previously described (Kiely *et al*, 2001). Composition data were transcribed from the product label. This methodology is considered valid for assessing supplement use (Patterson *et al*, 1998; Kiely *et al*, 2001).

6.2.1.3 Compilation of the food groups

Seven major food groups contributing to the mean daily intake of phylloquinone were derived from nineteen large food groups previously devised by the Irish Universities Nutrition Alliance (IUNA, 2001) as follows: Alcohol was excluded as it made no contribution to the mean daily intake of phylloquinone. Bread and rolls, and biscuits, pastries and cakes were all aggregated into one food group. Eleven Irish Universities Nutrition Alliance food groups that contributed <5% to the mean daily intake of phylloquinone were aggregated to form the 'Other' group: eggs and egg dishes; grains, rice, pasta and savouries; milks and yoghurts; cream, ice cream and chilled desserts; non-alcoholic beverages; cheeses and fat spreads; breakfast cereals; fruit and fruit juices; nuts, seeds, herbs and spices; fish and fish products; sugar, confectionery, preserves and savoury snacks; and nutritional supplements. The remaining five food groups were retained: vegetable group; potato group (including chipped and fried potatoes); meat group; dairy and fat spreads; soups, sauces and miscellaneous foods.

6.2.1.4 Assessment of adequacy of phylloquinone intake

As no Estimated Average Requirement (EAR) has been established for vitamin K in adults it was not possible to use the cut-point method of Carriquiry (1999) to estimate the prevalence of inadequacy of dietary intake. However, the US Food and Nutrition Board has recently established an adequate intake of 90 and 120 μg/d for adult women and men, respectively (IOM, 2001), based on reported intakes in apparently healthy US population groups. In the UK and EU, the recommended intake for phylloquinone is 1 μg/kg body weight per d (DOH, 1991; Scientific Committee for Food [SCF], 1993), based on its classical role in blood clotting. Therefore, to gain some measure of the adequacy of phylloquinone intakes, the proportions of the population not meeting the UK/EU recommendation and consuming the US adequate intake were calculated. The Tolerable Upper Intake Level (UL) is the maximum chronic daily

intake of a nutrient that is unlikely to pose a risk of adverse health effects to humans (SCF, 2000). There is no UL set for vitamin K (SCF, 2000).

6.2.2 Laboratory analysis

6.2.2.1 Serum carboxylated and undercarboxylated osteocalcin.

The concentrations of serum carboxylated-osteocalcin (cOC) and under-carboxylated osteocalcin (ucOC) were measured using ELISA (Gla and Glu EIA kits, Takara Biomedical Group, Otsu, Shia, Japan). The intra-assay CV for ucOC and cOC was 4.4% and 4.5%, respectively. %ucOC has been suggested as a key biochemical index of vitamin K status (Gundberg *et al*, 1998; Sokoll & Sadowski, 1996). The %ucOC was calculated by expressing serum ucOC as a percentage of total osteocalcin values (serum ucOC + serum cOC) as described recently (O'Connor *et al*, 2014). At the time of this work, serum was available for analysis of serum cOC and ucOC for 692 participants within the NANS sample.

6.2.2.2 Serum C-terminal telopeptide of type I collagen.

Serum C-terminal telopeptide of type I collagen (CTx) was measured using the Serum CrossLaps® ELISA kit (Nordic Bioscience Diagnostics A/S, Denmark). The intra-assay CV was 5.0%.

6.2.3 Data and statistical analysis

Statistical analysis of the data was conducted with PASW (version 21, for Windows; SPSS Inc.). Distributions of all variables were tested with Kolmogorov-Smirnov tests. Descriptive statistics on phylloquinone intakes, including mean, SD, median, IQR, and 5^{th} and 95^{th} percentiles, were determined from all sources (i.e., food sources and supplements) as well as food sources only, using the daily average method: phylloquinone intakes from each food and supplement reported were quantified by multiplying the weight of the food (grams) by the phylloquinone content (micrograms per 100 g). Phylloquinone intakes for the total population were also stratified by sex and age group (i.e., 18–35-y, 36–50-y, 51-64-y and ≥65-y). Dietary phylloquinone (from all sources and food-sources only) were not normally distributed and, thus, were log transformed to achieve near-normal distributions. Differences in phylloquinone intakes between men and women were compared using independent *t*-tests, and differences between age groups were compared using one-way ANOVA using either Tamhane, if the Levene test for equality of variance was not satisfied, or Scheffe's *post hoc* test.

Phylloquinone intakes in this study were derived from daily average intakes from 4 consecutive recording days. When using repeated short-term measurements of intake, such as 24-h food records, the variance of reported intake is inflated by the day-to-day variation of dietary intake within individuals (Willett, 1998). To estimate usual intakes of a nutrient, the within subject variation must be eliminated by an appropriate statistical method. Along with the daily average method, the National Cancer Institute (NCI) method (Tooze *et al*, 2010) was applied to NANS data and a usual intake estimate was calculated for the total population and stratified by sex. The NCI method was not applied to age groups because of the relatively smaller numbers of participants in these subgroups. The NCI-method was applied using SAS macros (version 2.1) which were downloaded from the website www.riskfactor.cancer.gov/diet/usualintakes/macro.html (date of download: July 2015). In the current analysis, we compared usual intake estimates based on the NCI method with intakes estimated from the daily average method.

Under-reporting of energy intake by self-reported dietary methods is well documented (Black *et al*, 1993; Livingstone *et al*, 1990). Energy under-reporters in NANS were identified by calculating basal metabolic rate and applying a Goldberg cut point of 1.1, as described previously (Goldberg *et al*. 1991; McGowan *et al*. 2001). We examined the impact of energy under-reporting on phylloquinone intakes by estimating median intakes when under-reporters were included and excluded.

The percent contribution of food groups to intakes of phylloquinone were calculated by the mean proportion method, as defined by Krebs-Smith *et al*.(1989) using SPSS© for Windows™ Version 20.0 (SPSS, Inc., IBM, Chicago, IL, USA). This method provides information about the sources that are contributing to the nutrient intake 'per person'. The mean proportion method is the preferred method when determining important food sources of a nutrient for individuals in the population group as opposed to investigating the sources of a nutrient within the food supply.

To allow for a comparison between the NSIFSC and NANS, those in the latter aged 18–64 y only were compared, because the NSIFSC did not include participants >64 y old. While we performed a statistical comparison of the mean daily intake estimates of phylloquinone among the two surveys (unpaired *t*-tests), it is important to stress that the survey sample sizes are sufficiently powerful to consistently observe statistically significant results even with marginal differences from a phylloquinone intake perspective.

Serum cOC, ucOC and %ucOC as well as CTx were not normally distributed and, thus, were log transformed to achieve near-normal distributions. Differences in serum %ucOC (the priority marker of vitamin K status; Shea & Booth, 2016) between men and women were compared using independent *t*-tests, and differences in %ucOC and ucOC between age groups were compared using one-way ANOVA using either Tamhane, if the Levene test for equality of variance was not satisfied, or Scheffe's *post hoc* test.

Pearson's correlations were performed between mean daily intake of phylloquinone and markers of vitamin K status (serum cOC, ucOC and %ucOC), and between mean daily intake of phylloquinone (and serum %ucOC) and serum CTx. Linear regression analysis was performed to test these associations while controlling for potential confounding factors such as age, sex, BMI, smoking, serum 25(OH)D, calcium intake, serum PTH, use of HRT/oral contraception, and total osteocalcin (marker of bone formation), as appropriate.

6.3 Results

6.3.1 Phylloquinone intakes.

The mean daily intakes of phylloquinone (μg) from all sources (i.e. food and supplements), and from food sources only, in men and women of different ages in NANS, which was a representative sample of the Irish adult population, are shown in **Table 1**. The mean daily intake of phylloquinone in the total group from all sources was 85.2 (SD 59.1) μg and from food sources only was 84.2 (SD 58.7) μg, showing that about 99% of the phylloquinone intake came from food. Median intakes of phylloquinone (from all sources) in men (71.8 μg/d) and women (70.2 μg/d) were similar ($P>0.2$). However, energy-adjusted median intakes (expressed as μg/10 MJ food energy) were significantly higher ($P<0.001$) in women than in men of all ages (102 and 79, respectively). In both men and women, phylloquinone intakes (μg/d from all sources, and from food sources only) were significantly lower ($P<0.05$) in the 18–35-year age group than in any of the other three age groups (i.e., 36–50 y, 51-64 y, and ≥65 y), with no differences between these latter three groups. This was also the case in women (Table 1); but in men, while the phylloquinone intake of the 18–35 y age group was also significantly lower ($P<0.05$) than that of the 36–50 y and 51-64 y age groups, it was similar to that of the ≥65 y (Table 1).

Table 1 Daily intakes of phylloquinone in Irish adults (aged 18-90 y), from all sources (food and supplements) and from food sources only as well as estimates of adequacy of intakes of phylloquinone

	All					Men					Women				
	All *n=1500*	18-35y *n=513*	36-50y *n=437*	51-64y *n=306*	≥65y *n=226*	All *n=740*	18-35y *n= 276*	36-50y *n= 205*	51-64y *n=153*	≥65y *n=106*	All *n=760*	18-35y *n= 255*	36-50y *n= 232*	51-64y *n=153*	≥65y *n=120*
Phylloquinone intakes (All Sources)															
Mean	85.2	75.7	86.4	97.8	87.7	86.0	79.8	86.7	97.9	83.4	84.4	71.3	86.2	97.8	91.5
SD	59.1	56.3	54.4	62.3	66.2	57.4	60.9	52.7	57.0	55.5	60.7	50.6	56.0	67.3	74.4
Median	71.2	62.2[a]	73.6[b]	82.9[b]	72.4[b]	71.8	64.5[c]	73.2[de]	83.2[d]	70.8[de]	70.2	59.4[f]	74.3[g]	82.6[g]	79.2[g]
5th percentile	25.1	23.2	28.5	30.3	20.5	25.1	23.6	28.7	30.9	19.1	25.0	21.2	28.0	29.2	26.8
95th percentile	194.4	171.6	186.8	239.5	193.9	188.9	171.9	187.5	213.0	197.1	196.8	172.6	190.8	250.4	195.3
Phylloquinone intakes (food sources)								µg/d							
Mean	84.2	74.6	85.7	97.0	86.7	85.3	78.9	86.3	96.9	83.1	83.2	69.9	85.2	97.1	90.0
SD	58.7	55.9	54.2	61.3	65.6	57.2	60.9	52.5	56.0	55.4	60.1	49.6	55.8	66.5	73.5
Median	70.8	61.1[a]	73.3[b]	82.6[b]	72.4[b]	71.6	62.4[c]	73.2[de]	83.2[d]	70.8[c,de]	69.5	58.3[f]	73.5[g]	82.0[g]	78.7[g]
5th percentile	25.1	22.8	28.5	30.3	20.5	25.1	23.6	28.7	30.9	19.1	24.9	20.3	28.0	29.2	26.8
95th percentile	192.8	171.4	186.8	235.2	190.5	188.1	171.9	187.5	206.4	197.1	195.5	169.0	189.1	249.3	190.8
Adequacy of phylloquinone intakes								%							
% >Adequate Intake	26.3	17.9	28.1	35.3	30.5	18.8	14.1	20.0	26.8	17.0	33.7	22.0	35.3	43.8	42.5
% <1 µg/kg body weight/d[1]	55.1	60.4	53.6	52.2	48.4	61.4	65.1	62.2	54.1	60.0	49.1	55.4	46.3	50.4	38.8

Medians in a row (within All, Men or Women) without a common letter differ, P<0.05 (either Tamhane, if the Levene test for equality of variance was not satisfied, or Scheffe's post hoc test) and P<0.0001 [1-factor ANOVA for both all sources and food sources only of vitamin K1 in All, Men and Women].

[1]Based on *n* of 1354, 495,399, 272, 188 (All); 658, 255, 185, 133 and 85 (Men); and 696, 240, 214, 139 and 103 (Women), respectively due to unavailability of body weight data for some participants.

Along with the daily average method, the NCI method (Chubb, 2012) was applied to NANS, with intakes stratified by sex, and phylloquinone intakes were compared with the daily average method. Intake estimates for the total NANS population were almost identical by the 2 methods (85.2 μg/d and 84.5 μg/d for daily average method and NCI method, respectively) but as expected there was a marked reduction in the SD using the NCI method compared to the daily average method (39.7 μg/d *v*. 59.1 μg/d, respectively). This was also the case when intake estimates for men and women, separately, from the two methods were compared (data not shown). The greatest difference was seen in the 95th percentile of intakes in women, for which the usual intake was 43 μg lower than the daily average intake.

Basal metabolic rate was used to identify under-reporters within the full NANS sample for which measured body weight data existed (*n*=1354). When under-reporters (29%) were included, median (IQR) phylloquinone intake was 71.2 (47.5-103.2) μg/d; when excluded, the intake was 76.8 (54.3-113.3) μg/d.

Mean daily intakes of phylloquinone in adults aged 18-64 y participating in the NANS (*n*=1274) and the NSIFCS (*n*=1379) are shown in **Figure 1**. Mean (SD) intakes of adults aged 18-64 y only increased very modestly between the NSIFCS survey in 1997-'99 and the NANS in 2008-'10 survey 79.5 (44.2) and 84.7 (57.8) μg/d, respectively].

6.3.2 Estimation of adequacy of vitamin K intakes.

Of the 1354 participants in NANS with measured body weight data, 55% had phylloquinone intakes below the UK and EU recommendation of 1 μg/kg body weight per d (DOH, 1991; SCF, 1993) (Table 1), while 29% (33% of men, 26% of women) failed to meet 67% (two-thirds) of this recommendation (data not shown). Furthermore, when phylloquinone intake data for full NANS (*n*=1500) were compared with current US adequate intakes (IOM, 2001), only 19% of men and 34% of women met or exceeded these intakes of 120 and 90 μg/d, respectively (Table 1).

6.3.3 Food sources of vitamin K.

The major food groups contributing to total intake of phylloquinone in men and women, aged 18-64 y, in NANS are presented in **Figure 2**. Overall, vegetables and vegetable dishes contributed 44% of the phylloquinone intake. Meat and meat products also made a significant contribution of 10%, while potatoes/potato products (primarily from the oils used in chips and fried potatoes) and dairy and fat spreads contributed 8% and 7%, respectively. These were in general similar to those reported in the NSIFCS of 18-64 y olds: vegetables and vegetable dishes (48%), potatoes/potato products (10%), dairy and fat spreads (10%), and meat and meat products (8%) (Duggan *et al*, 2004).

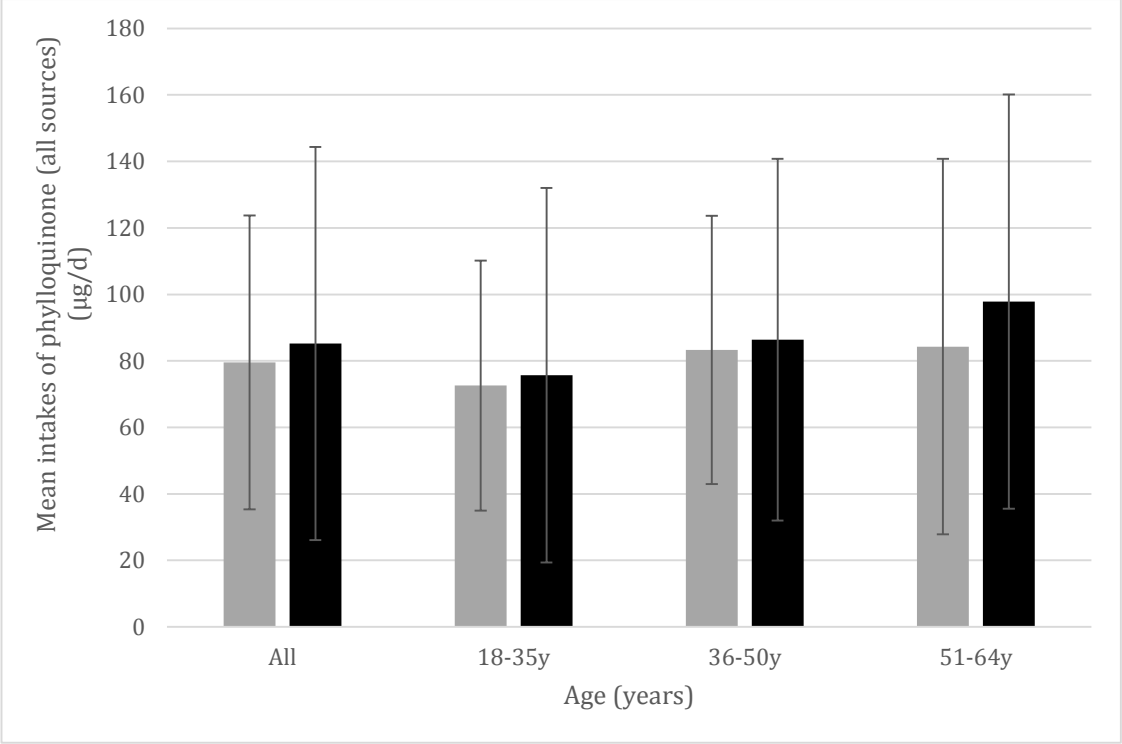

Figure 1. Phylloquinone intakes (from all sources) in all adults (aged 18-64 y), and stratified by age, who participated in the National Adult Nutrition Survey (NANS; *n*=1274) in 2008-'10 (black bars) and North/South Ireland Food Consumption Survey (NSIFCS; *n*=1379) in 1997-'99 (grey bars). Bars and error bars represent means ± SDs for *n* = 153–760.

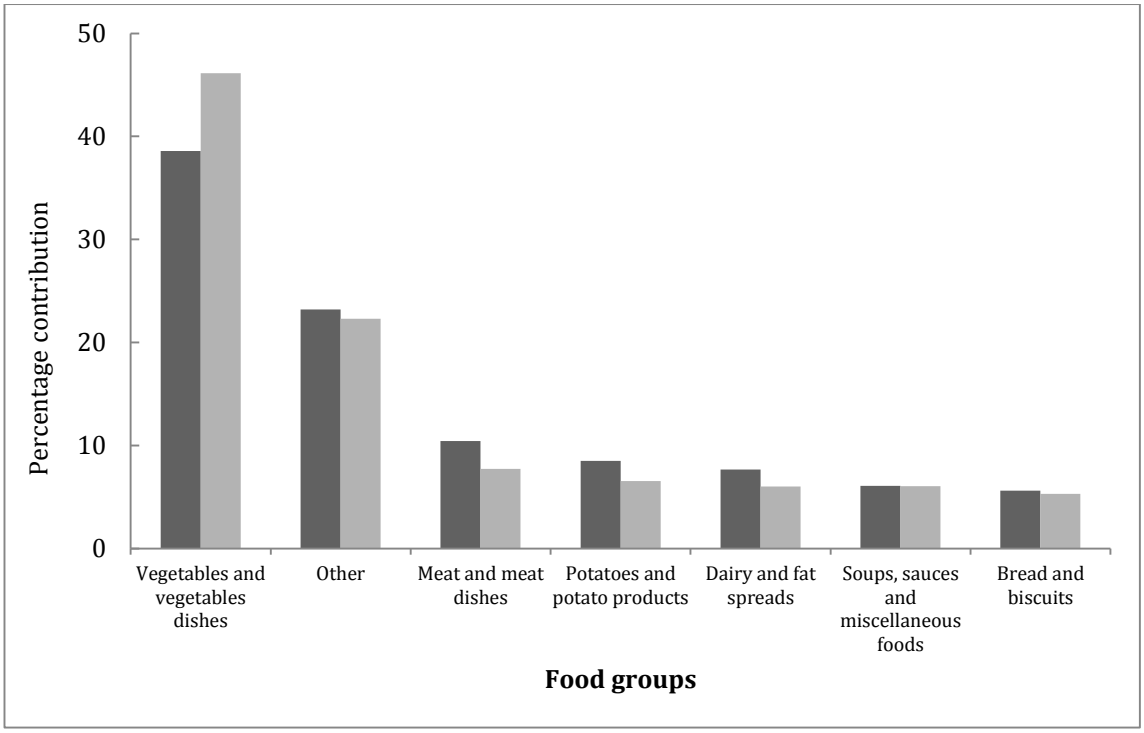

Figure 2. Percentage contribution of main food groups to the mean daily intake of phylloquinone in Irish men (*n*=634; dark bars) and women (*n*=640; grey bars), aged 18-64 years.

The major food groups contributing to total intake of phylloquinone in men and women aged ≥65 y old in NANS were vegetables and vegetable dishes (49%), dairy and fat spreads (12%), meat and meat products (6%), and potatoes/potato products (4%).

6.3.4 Vitamin K status markers

The biochemical markers of vitamin K status in men and women of different ages in NANS are shown in **Table 2**. There was no significant difference in demographic features of the group of participants for whom we had a blood sample to undertake vitamin K status analysis (*n* 692)and those in the entire sample ($P>0.1$; data not shown). Median serum %ucOC in men (40.0%) and women (40.6%) were similar ($P>0.3$). In both men and women, as well as in both sexes separately, median serum %ucOC was significantly higher ($P<0.05$ in all cases) in the 18–35-year age group than in any of the other three age groups (i.e., 36–50-y, 51-64 y, and ≥65 y), with no differences between these latter three groups (Table 2). Serum ucOC has also been reported separately as a measure of vitamin K status (Szulc *et al*, 1993; Duggan *et al*, 2004; Bügel *et al*, 2007; Yamauchi *et al*, 2010). In general, median serum ucOC was significantly higher ($P<0.05$ in all cases) in the 18–35-year age group than in any of the other three age groups (i.e., 36–50-y, 51-64 y, and ≥65 y), with no differences between these latter three groups (Table 2).

6.3.5 Associations between vitamin K intake and status markers, and with a marker of bone resorption.

The mean daily intake of phylloquinone was positively associated with serum cOC and inversely with serum ucOC and %ucOC (r = 0.095, -0.114 and -0.164, respectively [all in log scale], $P<0.02$ in all cases; n= 692). There was only a trend ($P=0.067$) for an inverse association between mean daily intake of phylloquinone and serum CTx (r = -0.070 [both in log scale]; n= 692). In a regression model adjusting for age, sex, BMI and smoking, the mean daily intake of phylloquinone (log) remained a significant determinant of serum %ucOC (also Log) [B = -0.694 (95% CI: -1.113, -0.275), $P=0.001$].

Serum %ucOC was positively associated with serum CTx (r = 0.196 [both in log scale]; $P<0.0001$; n= 692) (**Figure 3**). In a regression model adjusting for age, sex, BMI, smoking, dietary calcium intake, serum 25(OH)D, HRT/oral contraception use, PTH, and total serum osteocalcin, serum %ucOC (log) remained a significant determinant of serum CTx (also log) [B = 0.078 (95% CI: 0.049, 0.107), $P=0.001$]. Interestingly, serum 25(OH)D was not significantly associated with serum CTx ($P>0.5$). The model explained 42% variability in serum CTx.

Table 2. Biochemical markers of vitamin K status in Irish adults, aged 18-90 years, and stratified by age-group and sex[1]

	All					Men					Women				
	All	18-35y	36-50y	51-64y	≥65y	All	18-35y	36-50y	51-64y	≥65y	All	18-35y	36-50y	51-64y	≥65y
	n=692	*n=230*	*n=234*	*n=143*	*n=85*	*n=335*	*n= 110*	*n=110*	*n=77*	*n=38*	*n=357*	*n=120)*	*n=124*	*n=66*	*n=47*
Serum ucOC, ng/mL	2.5 (22.9)	3.3 (22.1)[a]	2.3 (22.9)[b]	2.3 (21.8)[b]	2.2 (10.5)[b]	2.6 (22.9)	3.6 (22.1)[c]	2.5 (22.9)[d]	2.0 (19.2)[d]	2.3 (9.4)[d]	2.5 (21.9)	3.2 (19.7)[a]	2.0 (19.4)[b]	2.6 (21.5)[ab]	1.9 (10.4)[b]
Serum cOC, ng/mL	4.1 (12.1)	4.3 (9.6)	3.8 (11.7)	4.2 (8.8)	4.4 (9.0)	4.3 (11.9)	4.6 (8.8)	4.3 (11.7)	4.0 (8.8)	4.4 (7.1)	3.9 (9.2)	4.1 (7.1)	3.5 (5.9)	4.8 (7.9)	4.2 (8.4)
Serum %ucOC, %	38.4 (79.5)	45.1 (78.5)[a]	35.5 (75.7)[b]	34.6 (71.1)[b]	32.5 (77.0)[b]	38.1 (78.9)	47.1 (78.5)[a]	35.1 (66.7)[b]	34.2 (71.1)[b]	35.1 (76.2)[b]	38.5 (76.9)	44.1 (70.0)[a]	36.0 (75.7)[b]	35.6 (64.1)[b]	32.1 (73.4)[b]

[1]Values are medians and interquartile ranges in parenthesis

Medians in a row (within All, Men or Women) without a common letter differ, P<0.05 (either Tamhane, if the Levene test for equality of variance was not satisfied, or Scheffe's post hoc test) and P<0.0001 [1-factor ANOVA for both all sources and food sources only of vitamin K1 in All, Men and Women].

ucOC; under-carboxylated osteocalcin; cOC, carboxylated-osteocalcin; %ucOC, under-carboxylated osteocalcin as a percentage of total (i.e., under-carboxylated osteocalcin plus carboxylated-osteocalcin)

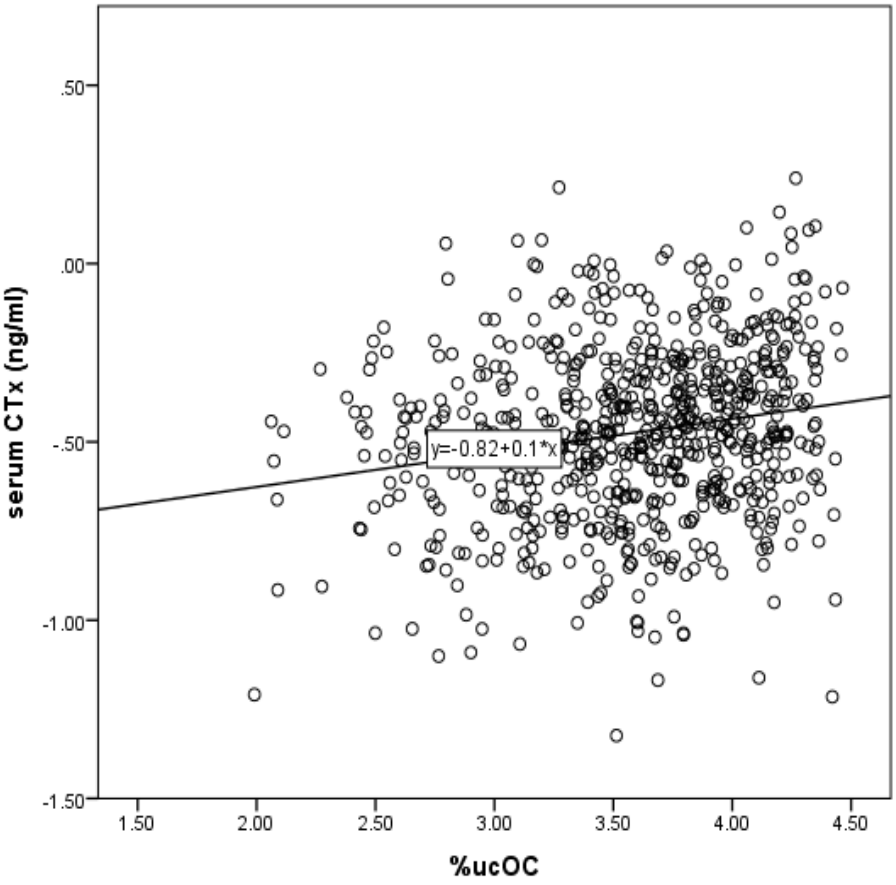

Figure 3. The association between percent serum under-carboxylated osteocalcin (%ucOC) and serum C-terminal telopeptide of type I collagen (CTx) [both in log scale] in Irish adults, aged 18-90 years (*n* 692) [y = 0.1x-0.82; *r*=0.196; *P*<0.0001].

6.4 Discussion

The present study estimated the phylloquinone intake, as well as, for the first time, assessed a biomarker of vitamin K status, in a representative population sample of Irish adults aged 18-90 y. The mean phylloquinone intakes of adults aged 18-64 y reported in the present study of 86 and 83 μg/d in men and women, respectively (85 μg/d for the entire group) were modestly higher than those reported by us previously for Irish adults aged 18-64 y (84 and 75 μg/d in men and women, respectively) (Duggan *et al*, 2004) and for values reported for Scottish men and women aged 25-65 y (76 and 69 μg/d, respectively; 73 μg/d for entire group) (Price *et al*, 1996). Likewise, in 1995, a 10-year follow-up study of the Scottish Heart Health Study participants estimated phylloquinone intakes of 54 μg/d for men and 56 μg/d for women (Bolton-Smith *et al*, 2000*b*).

The mean phylloquinone intakes of older adult men and women aged 65-90 y reported in the present study of 83 and 92 μg/d, respectively (88 μg/d for the entire group) were similar to that reported for elderly men in the UK (84 μg/d), but higher than that for women, and entire group, in the UK (73 and 78 μg/d, respectively) (Thane *et al*, 2002). Reported phylloquinone intakes of other older European populations are limited and, in some cases, have used different dietary tools that preclude comparison with the present data. For example, in The Netherlands, Schurgers *et al*. (1999) reported mean daily phylloquinone intakes of 250 μg/d for adults aged 55 y and older in the Rotterdam study; however, these intake data were obtained using a food-frequency questionnaire (FFQ). As the FFQ can tend to estimate higher vegetable intakes than a food diary method, phylloquinone estimates from these two assessment tools should not be compared.

Some studies have reported a decrease in phylloquinone intake with age, but especially in adults over the age of 65 years (Schurgers *et al*, 1999; Thane *et al*, 2002). However, in the present study, the younger adults (aged 18–35 years) had a lower mean phylloquinone intake than adults (either those aged 36–50 or 51-64 years) and older adults (65-90 years), and likewise serum %ucOC was higher in 18–35 y-olds than any of the other three age groups. The lower vitamin K nutritional status may be due to the lower vegetable consumption of younger Irish adults compared with over-35-year-olds, previously reported by O'Brien *et al*. (2003). In the present study, an investigation of the major food groups contributing to mean daily intake of phylloquinone showed that while vegetables and vegetable dishes were the main dietary sources of the vitamin, they made a greater contribution in ≥65 y-olds than 18-64 y-old (at 49% [43 μg/d] and 44% [37 μg/d]). Booth & Suttie (1998) reviewed eleven vitamin K intake studies in different population groups and found that studies showed

consistently higher intakes in older adults than younger adults, most probably due to lower green vegetable consumption in younger adults. Green vegetables alone (including lettuce) contributed 25% and 33% to the total intake, in 18-64 y olds and ≥65 y-olds, respectively. While potatoes and meat contain relatively little phylloquinone (~0.9 and 2.3 μg/100 g, respectively), in the present study, the meat group and potato group (including chipped and fried potatoes) contributed significantly to mean daily intakes of phylloquinone (about 10-18%, depending on age-grouping) due to the considerable quantities consumed and to the contribution from vegetables consumed in meat dishes. Thane *et al*. (2006) compared geometric mean phylloquinone intakes of adults aged 16-64 years from two national nutrition surveys in the UK (1986-7 and 2000-1) and reported a significant decrease (72 μg/d to 67 μg/d; $P<0.0001$), consistent with a concomitant decline in leafy green vegetable consumption. In contrast, the present study showed that contribution of green leafy vegetables to mean daily intake of phylloquinone in adults aged 18-64 y in Ireland remained relatively static from 26% in 1997-99 to 25% in 2008-'10, while mean daily intake of phylloquinone marginally increased (79 μg/d versus 85 μg/d, respectively).

Dietary supplements made essentially no meaningful contribution to the mean daily intake of phylloquinone at a population level (only 1 μg additional intake from all sources compared to food sources only). This agrees with data from adults (aged ≥19 y) who participated in NHANES 2003–2006 in the US which showed that the mean intake of vitamin K from food sources (including enriched/fortified) was 88.2 μg/d and increased only marginally to 94.7 μg/d when that from dietary supplements was accounted for (Fulgoni *et al*, 2011).

Without an EAR value, it is difficult to draw firm conclusions on the degree of inadequate intakes of vitamin K within the population. Only 19% and 34% of women and men, respectively, met or exceed their sex-specific AI values for vitamin K and thus had a high probability of having adequate intakes, while the proportion with inadequate intakes is less certain. Over a half of the entire population of adults had intakes which were less that the UK and EU suggested 1 μg/kg body weight per d, derived on the basis of coagulation (DOH, 1991; SCF, 1993), and in fact about a third and quarter of men and women, respectively, failed to meet ever two-thirds of this recommendation. Beyond the AI values for vitamin K, there is some evidence that even higher intakes are beneficial for bone health. For example, in the Nurses' Health Study, phylloquinone intakes less than 109 μg/d were associated with an increased risk of hip fracture (Feskanich *et al*, 1999). In the Framingham Heart Study, elderly men and women in the highest quartile of phylloquinone intake (median 254 μg/d) had a significantly lower adjusted relative risk of hip fracture than did those in the lowest quartile

of intake (median 56 μg/d) (Booth *et al*, 2000). While phylloquinone intake is associated with lower risk of hip fracture in the majority of observational studies, associations with bone mineral density (BMD) are less consistent [see review by Booth (2009)].

In the present study, while there was only a trend towards an inverse association between phylloquinone intake and serum CTx, serum %ucOC was positively associated with serum CTx, suggesting that as vitamin K status dis-improved, the rate of bone resorption increased. An increased rate of bone turnover in adults may be a risk factor for fracture (Riggs *et al*, 1996), because it exacerbates bone loss (Hansen *et al*, 1991). High rates of bone turnover are also associated with a disruption of the trabecular network, leading to a loss of connectivity which is not necessarily reflected in a decrease of bone mass (Parfitt, 1984). Yamauchi *et al*. (2010) showed a significant positive association between serum ucOC and urinary NTx (another biochemical marker of bone resorption) in 221 healthy Japanese women (31 pre- and 190 post-menopausal), even when adjusted for age, height, body weight and vitamin K intake. Interestingly, there was no significant association between serum ucOC and BMD at any site in the postmenopausal women (Yamauchi *et al*, 2010). There was a moderately strong inverse association (r=-0.259; P<0.001) between serum ucOC and vitamin K intakes in the 221 healthy Japanese women in that study (Yamauchi *et al*, 2010), whereas the inverse association, while evident, was weaker (r=-0.114; P=0.003) in the 692 NANS adults in the present work. The mean vitamin K intakes (both phylloquinone and MKs, as assessed by FFQ) in the Japanese women were 260 μg/d, whereas the mean phylloquinone intake in the present study (as assessed by 4 d food diary) was only 85 μg/d, which may have influenced the strength of the associations. It is important to note that serum ucOC and %ucOC are also influenced by a number of factors other than vitamin K intake, including waist circumference, season, HDL cholesterol, smoking, menopausal status and use of HRT (Shea *et al*, 2009). The inverse association between dietary vitamin K intake and serum ucOC has also been shown to depend on a functionally relevant allelic variant of the vitamin K epoxide reductase gene (Nimpstch *et al*, 2009).

Booth (2009) has recently suggested that one of the challenges in many observational studies is the inability to isolate the physiological effects of vitamin K deficiency from those of overall poor nutrition. Furthermore, she suggested that since the primary source of phylloquinone is green leafy vegetables, as it was in the present study, any positive associations between vitamin K intake and skeletal outcomes may just be reflective of generally healthier diets (Booth, 2009). While this is certainly a limitation in the present population study, the statistical analysis of serum %ucOC and serum CTx was adjusted for dietary calcium, serum 25(OH)D

and BMI amongst other non-nutritional-related factors. While the findings are based on analysis of a nationally representative population study, which is a key strength of the work, it is important to stress that, as such, causality between vitamin K nutritional status and the rate of bone resorption can't be inferred. Finally, as the UK food composition data used in the present study is largely based on phylloquinone content of foods (FSA, 2002), the contribution of MKs to total vitamin K intake and vitamin K status in Irish adults remains unknown.

In conclusion, despite being regarded as an at-risk population group, phylloquinone intakes in the elderly were not any worse than younger adults and, in fact, younger adults (aged 18-35 y) appear to be at a higher risk of inadequacy due to lower than average intakes of green vegetables. Irrespective of age-group, habitual intakes of phylloquinone in a significant portion of Irish adults may not be adequate based on comparisons with the 2001 US AI values (IOM, 2001), but also in relation to maintaining bone health (Feskanich *et al*, 1999; Booth *et al*, 2000), a suggestion supported by the findings of the present study which showed an association with a biochemical marker of bone resorption. Strategies to increase phylloquinone intakes include increasing consumption levels of green vegetables, and/or food fortification.

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Chapter 7

General Discussion

The drivers and motivation behind the work undertaken in this PhD thesis can be summed up by the following:

"One in every eight Europeans has vitamin D deficiency", according to University College Cork-based coordinators of the European Commission-funded ODIN vitamin D project'

"Knowing is not enough; we must apply. Willing is not enough; we must do"

- Goethe

Thus, the main objectives in relation to vitamin D in this thesis were to address key knowledge gaps in terms of:

- calcium and vitamin D interactions in the body and how these may influence dietary vitamin D requirements;
- devising creative food-based solutions (particularly, bio-fortification approaches) for the prevention of vitamin D deficiency that are sustainable and effective;

Separately, in light of ongoing international concern in relation to vitamin K status of many population groups, an additional main objective of the thesis was:

- to gain some perspective on vitamin K nutritive status in a nationally representative sample of Irish adults.

To achieve these objectives, we aimed to elucidate the relationship between habitual calcium intakes and the dietary vitamin D requirement through a combination of *post-hoc* analysis of our previous placebo-controlled, dose-related, vitamin D RCTs (randomised controlled trials) (Cashman *et al*, 2008; Cashman *et al*, 2009; Cashman *et al*, 2011a), perform an update of our previous meta–analysis (Cashman *et al,* 2011b), and then ultimately conduct a *de novo,* specifically designed, RCT of potential vitamin D – calcium interaction, which was informed by the *post-hoc* analysis. Following this, in an effort to inform options for the modification of the current food supply to deliver an increased content of vitamin D, several animal feeding trials were conducted to determine the feasibility of bio-fortification of animal-based foods with vitamin D, followed by consumer acceptability trials of resulting produce, and, finally a specific food-based intervention in free-living adults to assess the effectiveness of bio-fortified eggs (as an exemplar vitamin D-biofortified food) in terms of protecting serum 25(OH)D in winter.

In a separate piece of work to gain a better understanding of vitamin K's role in health and disease in the Irish population, we aimed to assess the phylloquinone intakes and measure

serum carboxylated and undercarboxylated osteocalcin concentrations, as measures of vitamin K status, in the National Adult Nutrition Survey (NANS) in Ireland, as a nationally representative sample. The association of vitamin K intake and status with a marker of bone turnover was also assessed to explore vitamin K-bone health relations in this population sample.

Overall, these objectives were met and the following sections will contextualize and summarize the key findings of the thesis, and highlight their relevance to potential stake-holders as well as possessing their own academic merit.

Dietary vitamin D requirements

There has been increased interest in the area of vitamin D in the last fifteen years that has prompted several regulatory bodies to re-evaluate their *Dietary Reference Intakes* (DRI) and *Dietary Reference Values* (DRV) for vitamin D (Cashman, 2015). As outlined in Chapter 2 of this thesis, these recommendations are extremely important tools in the development of public health policy to address the clearly evident and worrisome prevalence of vitamin D deficiency in the population. DRV/DRI reports have highlighted key knowledge gaps, which they identified in the respective processes that need to be addressed not only to inform and enhance the next iteration of vitamin D requirement estimates, but also in order to ultimately produce an effective and sustainable policy that will promote adequate intakes of vitamin D in the population. In this regard, a key knowledge gap identified by the Institute of Medicine (IOM) in their 2011 report on calcium and vitamin D, and by ourselves in light of the close interaction of these two nutrients (Cashman & Kiely, 2011) was a better understanding of the influence of habitual dietary calcium intakes on the regulation and catabolism of serum 25(OH)D.

The vitamin D DRI established by the IOM in 2011, as per convention, was based on the assumption that the requirement for dietary calcium was being achieved (IOM, 2011), however their report highlighted the potential for inadequate calcium intakes to cause changes in the efficient handling of, or physiological response to, vitamin D that might not otherwise be present. Considering a significant portion of adult populations in Europe and North America fail to meet respective dietary calcium requirements (Flynn *et al*, 2009; IOM, 2011; IUNA, 2011), and that both the 2010 and 2015 *Dietary Guidelines Advisory Committee* in the US identified four nutrients of public health concern with calcium (and vitamin D) being one of them, clarification of whether inadequate intakes of calcium increase the dietary requirement for vitamin D was of significant importance from a public health perspective, both in terms of informing various DRV exercises in Europe following on from IOM and also

in devising preventative strategies for vitamin D deficiency. In the UK, the Department of Health commissioned a research project to address this key knowledge gap, so as to inform its ongoing re-evaluation of vitamin D DRV, but also the associated risk management options that may stem from that (e.g. vitamin D supplementation and fortification). The *Vitamin D Research Group* at University College Cork successfully competed for this commissioned research and the work conducted formed the basis of my Chapter 3 in the present thesis. We aimed to clarify the inter-relationship of calcium and vitamin D in the body in terms of an influence on dietary vitamin D requirements using a three-pronged approach:

Firstly, *post hoc* analysis of data from four placebo-controlled, dose-related, individual vitamin D RCTs (Cashman *et al*, 2008; Cashman *et al*, 2009; Cashman *et al*, 2011a) suggested very little, if any, effect of habitual calcium intake on the response of serum 25(OH)D to vitamin D intake (and thus vitamin D requirements). However, it is important to note, that none of the four previously-published vitamin D RCTs were specifically designed to investigate to impact of dietary calcium level and accordingly had relatively small numbers of subjects with calcium intakes below the lower cut-offs of 550 mg/d [EU Estimated Average Requirement (EAR)] and 700 mg/d [UK Reference Nutrient Intake (RNI)] (Department of Health, 1991; Scientific Committee on Foods, 1993), which are important public health-related intakes. This was an important limitation in terms of drawing conclusions on the impact of dietary calcium on response of serum 25(OH)D and thus dietary requirement estimates for vitamin D.

As an additional approach which favoured inclusion of a wider collection of studies, our update of our previous meta-regression approach (Cashman *et al*, 2011b) using data from a mix of winter-based RCTs in children (*n* 2 RCTs), adults (up to 50 y; *n* 4 RCTs) and older adults (>50 y; *n* 5 RCTs) suggested an interaction between the response of winter serum 25(OH)D to total vitamin D and dietary calcium category (i.e., group mean above or below a threshold of 1092 mg/d). Using a target serum 25(OH)D of 50 nmol/L (which the IOM suggest covers the needs of 97.5% of the population in terms of bone health), then those with a higher mean calcium intakes (≥1092 mg/d) required a lower vitamin D intake compared to those with lower mean intakes (<1092 mg/d). It is important to stress that the 1092 mg/d cut-off was a median of group mean calcium intakes and thus reflected more a delineation of high calcium intake from lower intake. It is possible that the significant interaction in this meta-regression approach may be related to the relatively unadjusted analysis. In the analysis of the four previous vitamin D RCTs some of the significant differences in response of serum 25(OH)D to different doses of vitamin D, especially in 64+ year olds became non-significant when adjusted for smoking, BMI, age and gender. Data on possible confounding variables were not available or sufficient in all the RCTs selected for the meta-regression in the present analysis

such that they couldnot be included. Overall, the ambiguity in the resulting data from this 1st prong of our investigation, reinforced the need for a *de novo* RCT that was specifically designed to address the hypothesis that calcium intake modifies vitamin D requirement (this formed the 2nd prong of our approach).

For this 2nd prong, we conducted a 15-week, winter-based, vitamin D_3 intervention study in 125 apparently healthy, free-living, white adults (aged ≥50 y) at a latitude of 51°N that examined the potential interactions of dietary calcium intake on both the decline of serum 25(OH)D over winter whilst on a habitual inadequate vitamin D intake, as well as, on the response of serum 25(OH)D to an intake aimed at achieving at least the IOM suggested 'RDA-like' 25(OH)D concentration of 50 nmol/L (IOM, 2011). Recent dietary requirement estimates for vitamin D from both sides of the Atlantic have prioritized winter-time as a critical period during which intakes of vitamin D should maintain serum 25(OH)D concentrations above chosen cut-offs (IOM, 2011; German Nutrition Society, 2012; Nordic Nutrition Recommendations, 2012; SACN, 2016; EFSA, 2016). A key finding of this RCT was that the responses in serum 25(OH)D concentrations throughout winter, as well as indices of vitamin D activation and catabolism as potential explanatory variables, were similar in older adults irrespective of whether they were on relatively low (<550/700 mg/d) or high habitual (>1000/1200 mg/d) calcium intakes. Further to this, and as the 3rd and final prong of our approach, we analysed a subset of bio-banked sera from the NANS in Ireland for serum 24,25$(OH)_2$D, the first metabolite produced in the pathway of vitamin D catabolism. Using existing data from the survey (NANS, 2011), we identified participants within the survey with highest and lowest calcium intakes, and high and low vitamin D status, and measured serum 24,25$(OH)_2$D in this subset. This analysis showed a lack of association between calcium intake and serum 24,25$(OH)_2$D in adults aged 18-84 years; which further supports the findings of the our *de novo* RCT (Cashman *et al*, 2014a).

Collectively, these new data from Chapter 3 suggest that recently proposed dietary requirement estimates for vitamin D in North America (IOM, 2011) and subsequently in a number of European member states (German Nutrition Society, 2012; Nordic Nutrition Recommendations, 2012; Health Council of the Netherlands, 2013; SACN 2016; EFSA 2016) will ensure the adequacy of serum 25(OH)D concentrations in older adults even when the calcium intakes of these adults are in the inadequate range (<700 mg/d, and even <550 mg/d). These findings will be of interest to the North American IOM in relation to filling one of its identified key knowledge gaps from their DRI and re-evaluations exercise (IOM, 2011). The outcome of this work is also reflected in the current SACN DRV report (SACN, 2016).

Beyond their direct relevance to agencies briefed with development of DRI/DRV, these findings also are important from a policy perspective in terms of devising preventative strategies for vitamin D deficiency. Strategies for bridging the gap between these new vitamin D requirement estimates and current intake of vitamin D in the UK, Ireland, US and elsewhere will not be dependent on habitual calcium intakes, at least for those with intakes >550 mg/d, which is reflective of habitual calcium intakes in the vast majority of Irish and UK adults [87% of Irish adults have habitual calcium intakes > 550 mg/d; (NANS, 2011)] The data will inform Irish and UK government, as well as European, policy on vitamin D supplementation and fortification of foods. Foods considered for fortification will not necessarily be required to also contain calcium (e.g. dairy produce), which will aid wider distribution of vitamin D throughout the food supply and accommodate diversity in the diet. Overall,these data helps contribute to a sound scientific basis for development of nutrition policy and programmes for ensuring adequate vitamin D status in the population.

As mentioned above, and outlined in detail in Chapter 2, DRI and DRV for vitamin D are important from a public health point of view, as they are designed by authoritative agencies to prevent vitamin D deficiency in the population and thus are the targets at which we aim our food-based solutions. The low dietary vitamin D intakes in European populations, as outlined comprehensively by Vinas *et al.* (2011), highlight the fact that the current food supply (even including current food fortification and vitamin D supplementation practices) does not provide enough to prevent wide scale low vitamin D status in Europe (Cashman *et al*. 2016). Recent reports from Canada and the US have also suggested that the current food supply is unable to provide sufficient protection against vitamin D deficiency (Calvo & Whiting, 2013). Thus, there is a pressing need for creative food-based solutions to increase the distribution of vitamin D intakes for prevention of vitamin D deficiency and maintenance of nutritional adequacy. However, persistent knowledge gaps hinder development and implementation of safe and effective food-based approaches to vitamin D deficiency (Kiely & Cashman, 2015). The work as outlined in Chapters 4 and 5 in this thesis aimed to address some of these gaps in terms of biofortification of eggs, pork and beef with vitamin D to improve the total vitamin D activity and to provide evidence of the effectiveness of vitamin D biofortified eggs, as one exemplar, in protecting against low vitamin D status in the population.

Vitamin D biofortification of eggs, pork and beef

Biofortification of food with vitamin D is a novel approach aimed at improving the dietary availability of vitamin D from the current food supply and by that means, increasing the distribution of vitamin D intakes in the population. Chapter 4 in the present work focuses on the biofortification of animal produce, such as eggs, pork and beef, with vitamin D (as vitamin D_3 and/or 25-hydroxyvitamin D_3). Previous research in this area was primarily focused on the impact of additional vitamin D on the welfare of the animal itself or the quality of its resulting animal produce (e.g., eggs and meat). Nevertheless, these older animal trials provided data on the potential of biofortification to increase the vitamin D content of the food supply. The difficulty with the evidence-base, however, was that the majority of these studies in hens, pigs or cattle included levels of vitamin D and/or HyD® (as commercially available 25-hydroxyvitamin D) in the feed which exceed the EU allowable maximum levels for use in respective feeds. Thus, the primary aim of the current research was to use levels of additional vitamin D and/or HyD® (where permissible) in the diets of hens, pigs and beef heifers that adhered to EU regulations, so as to provide key quantitative data on vitamin D content and total activity of the resulting foodstuffs, as well as new data on the sensory acceptability of these vitamin D biofortified foods. These are key data underpinning the development of bio-fortified foods suitable for human consumption.

In relation to vitamin D biofortification of eggs, we had three key research questions:

1. Using levels of addition of vitamin D and/or HyD® in the feed which did not exceed the EU allowable maximum levels for use in laying hen feed (equivalent to 3000 IU/kg feed; EFSA, 2009, EFSA, 2012), what increases in the content of vitamin D_3 and 25-hydroxyvitamin D_3 and in total vitamin D activity in the resulting eggs were possible?
2. Did these vitamin D biofortified eggs have a good consumer acceptability profile?
3. What was the impact of consumption of seven of these vitamin D biofortified eggs weekly on vitamin D status of older adults during two months of winter?

Importantly, as outlined in Chapter 4, the findings of our hen-feeding trials suggest that the addition of vitamin D and/or HyD® at levels that adhere to the EU maximum allowable levels can increase the total vitamin D activity of an egg to ~5 μg/whole egg (or half that of the IOM's EAR of 10 μg/d). Consumer taste panel trials confirmed that these vitamin D biofortified eggs had full consumer acceptability and no deterioration in any sensory characteristics. Importantly, Chapter 5 provided the first RCT evidence, to our knowledge,

that consumption of seven of these vitamin D biofortified eggs per week, which does not conflict with healthy eating guidelines, helped to maintain serum 25(OH)D concentrations over winter months, and prevented the occurrence of vitamin D deficiency, as evidenced in a control group who consumed up to 2 commercial eggs per week.

In countries that do not implement mandatory fortification of milk, meat (such as pork and beef) can be a significant source of vitamin D in the diet (Hill *et al*, 2008). Importantly, pork and beef naturally contain small amounts of 25-hydroxyvitamin D in addition to vitamin D, which contribute to their overall total vitamin D activity. The current work (Chapter 4) has shown that the total vitamin D activity of these meats can be increased following biofortification with vitamin D and/or HyD® (where permissible) at levels that adhere to EU regulations. The total vitamin D activity of lean pork meat appears to be in the range 1.0 to 2.9 μg/170 g meat (which is an average serving size) based on data from the present work and that from another study of pigs by Burild *et al*. (2016). In fact, the total vitamin D activity of less lean pork products could be even higher, as Burild *et al*. (2016) have shown that the total vitamin D activity of fat from animals fed HyD® or vitamin D_3 (2000 IU/kg feed) was 24.0 and 17.7 μg/kg, respectively. In addition, the total vitamin D activity of beef (rib eye) steak appears to be around 1.5 μg/144 g meat (average serving size). Importantly, these small to moderate increases made to the total vitamin D activity of pork and beef, following the addition of vitamin D_3 and/or HyD® to animal feedstuffs, had no adverse effect on consumer acceptability, based on the findings of the sensory panel trials as outlined in Chapter 4. In fact, there was some suggestion of the overall consumer acceptability of beef steaks being higher in animals receiving 2000 or 4000 IU vitamin D_3/kg diet compared to no addition to the feed. This has implications potentially for vitamin D content of beef from entirely grass-feed animals versus those who receive a finishing diet containing vitamin D in the mix.

Overall, this work adds to the evidence-base that vitamin D biofortified eggs and meats can be produced with enhanced vitamin D content and also maintain consumer acceptability. The new data on the impact of vitamin D biofortified eggs on vitamin D status in particular has public health relevance in light of the percentage of the population with vitamin D deficiency in winter (Cashman *et al*, 2013), but also as one in two adults are reported to include eggs or egg dishes in their diet on a regular basis (NANS, 2011).

Determination of vitamin D and 25-hydroxyvitamin D in foods: importance to public health nutrition and also methodological issues to be aware of for future work

It is important to stress that the calculation of total vitamin D activity of the eggs and meat in the present work in this thesis applies a conversion factor of 5 for 25-hydroxyvitamin D, based on RCT data presented by us previously (Cashman *et al*, 2012) and is that used in several Food Composition tables, including the McCance & Widdownson's *The Composition of Foods* in the UK (FSA, 2008; Zurich, 2010; Saxholt *et al*, 2008). For example, this underpinned our estimates that the Vitamin D_3-eggs and HyD®-eggs groups in our RCT in Chapter 5 received an additional 3.5 and 4.5 μg/d of vitamin D, respectively, which together with the their habitual dietary vitamin D yielded a mean daily intake of ~10 μg/d. There is ongoing discussion on whether this factor 5 may be used in the calculation of total vitamin D for nutrition labeling of foods, and may differ country by country. Nevertheless, Chapter 5 in the present work again highlighted the potential underestimation of vitamin D intake in estimates from countries where the contribution of 25-hydroxyvitamin D to total vitamin D content of animal-based foods is not accounted for in the food compositional tables. For example, the current USDA National Nutrient Database for Standard Reference suggests the vitamin D content of 100 g raw egg is 2 μg, an estimate which does not include 25-hydroxyvitamin D. Accordingly, vitamin D intake estimates from NHANES in the US do not account for the contribution of 25-hydroxyvitamin D (Fulgoni *et al*, 2011). Importantly, Taylor *et al*. (2014) recently performed some modelling to include overall food-derived 25-hydroxyvitamin D in intake estimates for US adults, which showed that there was a potentially meaningful increase (1.7-2.9 μg or 15-30% of the EAR) in vitamin D intake estimates.

The issue of assessment of vitamin D, and its metabolites, in foods is an important one overall. As mentioned in Chapter 2, evidence of effectiveness of food fortification (including biofortification) approaches from RCTs which evaluate their impact on reducing the prevalence of vitamin D deficiency in the populations studied is a key priority, but also dietary modelling analysis based on data from nationally representative dietary surveys can provide *in silico* projections of how these food interventions may impact on the degree of vitamin D intake inadequacy in the population (Cashman & Kiely, 2011). Such dietary modelling whichassesses the risk of inadequate or excessive intakes of vitamin D in the population, is reliant on accurate and up-to-date food composition data. It is difficult for the various food composition databases to consistently provide accurate information as regards the provision of vitamin D in the diet as a result of discretionary fortification and to stay abreast of new

innovative strategies, such as biofortification. However, the food composition databases do not have a standardised approach to reporting vitamin D compounds, in particular 25-hydroxyvitamin D (Taylor *et al*, 2014), which should be addressed to prevent confusion arising.

Accurate representation of the vitamin D content of the current dietary supply is further compounded by the lack of a standardised method for the determination of vitamin D and 25-hydroxyvitamin D in food. It has been suggested that an accurate database is dependent on accurate analytic methods for food (Byrdwell *et al*, 2008). The method for the measurement of vitamin D compounds in food is continuously being revised to optimise this time-consuming analysis. However, due to the nature of the method involved, a low number of laboratories undertake this type of analysis, and even in those that do, the between-laboratory variability in reported vitamin D, but even more so 25-hydroxyvitamin D, content is large. In this regard, the development of control and reference materials is particularly important, as currently it is not possible to determine whether variability in results should be attributed to real differences in food composition or differences in analytical methods and skill in assay performance. Furthermore, it has been suggested that differences in matrix and analyte levels may affect the assay performance even if the same methodology is used, again highlighting the pressing need for use of control and reference materials across a wide range of foods (Byrdwell *et al*, 2008; Roseland *et al*, 2016). Importantly, a recent vitamin D round-robin analysis of five different foods by Roseland *et al*. (2016) has paved the way for the development of reference materials for use in food analyses and data from that study will be used to assign reference values for vitamin D_3 and 25(OH)D_3 in several NIST standard reference materials in the US, which will come on stream in the near future. This advancement will aid further research in determining amounts of vitamin D and 25(OH)D in foods (and supplements) to provide accurate and precise food composition data.

How analysis of other vitamin D metabolites is of importance in informing the world of vitamin D nutrition

In an aside to the main objective of Chapter 3, additional LC-MS/MS analysis on samples utilised in that work showed that serum 24,25$(OH)_2$D may be responsible for the positive bias observed in some commercial immunoassays for the measurement of serum total 25(OH)D, relative to LC-MS/MS derived estimates of 25(OH)D. We showed that this is likely due to the cross-reactivity of the anti-bodies utilised in some immunoassays with serum 24,25$(OH)_2$D. In addition, the research also suggests that serum 24,25$(OH)_2$D may be potentially informative in terms of vitamin D status (Cashman *et al*, 2015). Assessment of serum 24,25$(OH)_2$D,

particularly when expressed as a molar ratio to 25(OH)D, has found favor as an index of vitamin D deficiency and catabolism in healthy individuals (Schoenmakers *et al*, 2010; Wagner *et* al, 2011; Cashman *et al*, 2015; Kaufmann *et* al, 2014), as well as in those with rare genetic mutations in the CYP24A1 gene (Schlingmann *et al*, 2011; Kaufmann *et* al, 2014). The ratio of $24,25(OH)_2D$ to 25(OH)D may also be an indicator of response to vitamin D supplementation (Wagner *et al*, 2011) and may help to explain some of the well-documented inter-individual differences in response of serum 25(OH)D to the same administered dose of vitamin D. DEQAS has very recently begun to collect $24,25(OH)_2D$ data on its regular quarterly serum cycles from those laboratories which assess it, highlighting the growing interest in this serum metabolite. The additional analysis of serum from the *de novo* RCT in Chapter 3, when combined with existing data from NANS on the serum concentration of 3-epimer of 25(OH)D (3-epi-$25(OH)D_3$), showed how the concentration of 3-epi-$25(OH)D_3$ increased with vitamin D supplementation and decreased in the placebo group over winter (Cashman *et al*, 2014b), pointing towards possibility that both dietary supply and dermal synthesis of vitamin D_3 contribute to serum 3-epi-$25(OH)D_3$ concentration.

Phylloquinone intakes and food sources in a representative sample of Irish adults

In a separate piece of this PhD's research programme, we aimed to gain some insight into vitamin K status in a nationally representative sample of Irish adults. As new roles for vitamin K emerge beyond coagulation (Booth, 2009), there is increased interest in measuring vitamin K status in population-based studies, particularly, nationally representative samples. In this regard, the present work (Chapter 6) estimated phylloquinone intakes and, in addition, for the first time assessed a biomarker of vitamin K status (percent undercarboxylated osteocalcin in serum [%ucOC]), in a representative sample of Irish adults aged 18-90 years. Mean phylloquinone intakes of adults (aged 18-64 y) reported in NANS (as our most current national nutrition survey of adults in Ireland) at 86μg/d and 83 μg/d in men and women, respectively, were only modestly higher than those reported by us previously for Irish adults (aged 18-64 y) in our previous survey of adults, ten years prior (84 μg/d and 75 μg/d in men and women, respectively) (Duggan *et al*, 2004). The mean phylloquinone intakes of older adult men and women (aged 65-90 y) in NANS were 83 and 92 μg/d, respectively. This is the first representative data of phylloquinone intakes in older Irish adults (>65 y), as the previous survey only included adults aged 18-64 y (Duggan *et al*, 2004). The main dietary contributors to mean daily intake of phylloquinone intakes in the Irish diet were green vegetables (25-33%), followed by the meat and potato group (10-18%) dependent on age group, with supplements providing little impact on vitamin K intakes.

Interestingly, other studies have reported a decrease in phylloquinone intake with age, but especially in adults over the age of 65 years (Schurgers *et al*, 1999; Thane *et al*, 2002). However, in the present study, the younger adults (aged 18–35 years) had a lower mean phylloquinone intake than adults (either those aged 36–50 or 51-64 years) and older adults (65-90 years), and likewise serum %ucOC was higher in 18–35 y-olds than any of the other three age groups. This may be a result of the distribution of vegetable intakes being skewed with age as previously reported by O'Brien *et al*. (2003).

It has been over a decade and a half since the IOM (2001) has considered vitamin K requirements and established AI values based on representative dietary intake data from healthy individuals in the US. An ongoing limitation in terms of assessing the degree of vitamin K inadequacy within the population is the lack of an EAR value, as well as non-defined thresholds for many of the biochemical markers of vitamin K status. Without an EAR value, it is difficult to draw firm conclusions on the degree of inadequate intakes of vitamin K within the population. However, the present work suggests that only 19% and 34% of women and men, respectively, met or exceeded their sex-specific AI values for vitamin K and thus had a high probability of having adequate intakes, while the proportion with inadequate intakes is less certain. Over a half of the entire population of adults had intakes which were less that the UK and EU suggested 1 μg/kg body weight per d, derived on the basis of coagulation (Department of Health, 1991; Scientific Committee on Foods, 1993), and in fact about a third and quarter of men and women, respectively, failed to meet even two-thirds of this recommendation.

As the UK food composition data used in the present study are largely based on phylloquinone content of foods (FSA, 2002), the contribution of MKs to total vitamin K intake and vitamin K status in Irish adults remains unknown. This is an area which may be addressed by collaboration with the limited number of laboratories, both in Europe and the US, who generate food MK compositional data and application of these data within NANS.

In conclusion, Chapter 6 showed that despite being regarded as an at-risk population group, phylloquinone intakes in the elderly were not any worse than younger adults and, in fact, younger adults (aged 18-35 y) appear to be at a higher risk of inadequacy due to lower than average intakes of green vegetables. Irrespective of age-group, habitual intakes of phylloquinone in a significant portion of Irish adults may not be adequate based on comparisons with the 2001 US AI values (IOM, 2001), but also in relation to maintaining bone health (Feskanich *et al*, 1999; Booth *et al*, 2000), a suggestion supported by the findings of

the present study which showed an association with a biochemical marker of bone resorption (serum CTx). Strategies to increase phylloquinone intakes include, increasing consumption levels of green vegetables, and/or food fortification. Interestingly, there are some preliminary data to suggest that biofortification of foods with vitamin K, akin to the vitamin D approach, may be feasible, and this an area of future research worth progressing.

Concluding remarks

In conclusion, this thesis provides key new data which will be of use to the IOM and other regulatory bodies in their future revisions of DRI and DRVs. In addition, considering the pressing need for creative food-based solution to bridge the gap between current intakes and these vitamin D recommendations, the present work contributes to our understanding of bio-fortified foods, as one subset of such food-based solutions, and provides the first RCT proof of efficacy for bio-fortified eggs as an exemplar in protecting against low vitamin D status in winter. Finally, it provides informative data on current phylloquinone intakes and for the first time, vitamin K status, in an Irish population, which suggests the need to explore strategies to improve vitamin K intakes in our population.

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